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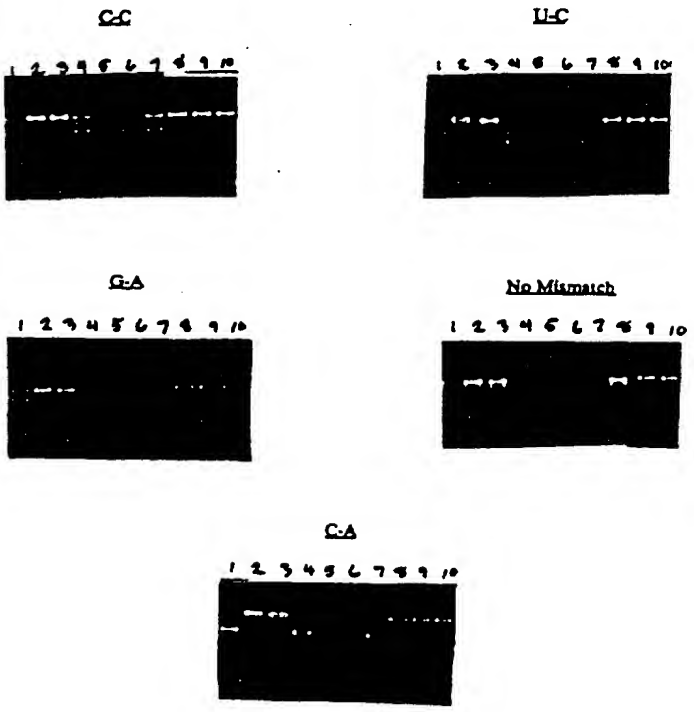
(57) Abstract

The present invention addresses compositions and methods for cleaving nucleic acids. The invention allows one to reliably detect point mutations in long and short target regions of nucleic acids in a safe, non-labor intensive, and cost effective manner. The methods and c mpositions of the present invention allow for the use of various RNases, such as RNase A, RNase I and RNase T1, in the detection of mutations. The present invention also identifies reaction conditions that result in significant improvement in specific mismatch cleavage in the NIRCA™ assay.



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DESCRIPTION

METHODS AND COMPOSITIONS FOR USE IN CLEAVAGE AND DETECTION OF BASE PAIR MISMATCHES IN DOUBLE-STRANDED NUCLEIC ACIDS

BACKGROUND OF THE INVENTION

U.S. Patent applications 08/371,531 and 08/534,977 are specifically incorporated herein without disclaimer. The U.S. Government may own rights in the present invention pursuant to U.S. grant number CA57045.

1. Field of the Invention

The invention relates to improved compositions and methods for the digestion of nucleic acids, specifically, to improved compositions and methods for cleaving base pair mismatches in double-stranded nucleic acid targets. The invention further provides novel components of the reaction mixture in which RNase is used to cleave nucleic acids and base pair mismatches.

2. Description of the Art

The digestion of nucleic acids has important applications in the field of molecular biology. For example, digestion of single base mismatches in RNA/DNA and RNA/RNA targets has been used to detect mutations.

Nucleases are enzymes used to digest nucleic acid. DNases degrade single and/or double-stranded DNA, RNases degrade single and/or double-stranded RNA, and non-specific nucleases such as SI degrade both RNA and DNA, with preference for single-stranded nucleic acid. Nucleases are enzymes that catalyze the hydrolytic cleavage of a

polynucleotide chain by cleaving the phosphodiester linkage between nucleotide residues. They can be classified as either exonucleases, which cleave nucleotides from the end of the chain, or endonucleases, which cleave from within the chain, and may specifically cleave single stranded or double stranded nucleic acids or both. Nucleases may also act only or preferentially on DNA, DNases, or RNA, RNases, or they may cleave both. Most nucleases cleave the nucleic acids without sequence specificity. However, some nucleases cleave specifically in a particular base or a specific sequence, such as restriction enzymes.

The use of nucleases to digest or cleave nucleic acids is well known in the field of molecular biology. For example, nucleases can be used to digest base pair mismatches that result from point mutations in genes. In the case of detecting mutations by digestion or cleavage the only nucleases which have been widely used are single-strand specific RNases; in particular, RNase A has been used for this purpose.

Methods for rapidly, reliably, and inexpensively detecting new point mutations have wide application in diagnosis and treatment of genetic diseases and cancer, and also in genetic counseling. These methods are of great benefit as well in basic research into the causes of a variety of human genetic diseases and in establishing human genetic linkage maps.

In most genetic diseases, the causative mutations are widely distributed over a large number of sites. Relatively few genetic disorders are caused by defined mutations at single sites; sickle cell anemia is one example of a disease phenotype that is always caused by the same specific mutation (*i.e.*, an A→T transversion at codon 6 of the beta-globin gene). More commonly, genetic diseases, especially cancer, are associated with a number of different mutations in different sites in different arrays of genes. An example of this is breast cancer. For instance, mutations in the recently identified BRCA1 gene, which is thought to be associated with familial breast cancer, are scattered throughout a

5 kilobase coding region. Dispersed mutations in several other genes, including *p53*, EGFR, IGR, and her2/neu, are also believed to play important roles in breast cancer.

Methods for genetic screening by identifying mutations associated with most genetic diseases and cancer must be able to assess large regions of the genome. Once a relevant mutation has been identified in a given patient, other family members and affected individuals can be screened using methods which are targeted to that site. The ability to detect dispersed point mutations is critical for genetic counseling, diagnosis, and early clinical intervention as well as for research into the etiology of cancer and other genetic disorders. The ideal method for genetic screening would quickly, inexpensively, and accurately detect all types of widely dispersed mutations in genomic DNA, cDNA, and RNA samples, depending on the specific situation. Currently there are no methods which achieve these goals.

Historically, a number of different methods have been used to detect point mutations, including denaturing gradient gel electrophoresis ("DGGE"), restriction enzyme polymorphism analysis, chemical and enzymatic cleavage methods, and others (Cotton, 1989). The more common procedures currently in use include direct sequencing of target regions amplified by PCR™ and single-strand conformation polymorphism analysis ("SSCP").

Direct sequencing of PCR™ products is considered to be the most reliable method for identifying new mutations. However, sequencing is also the most expensive and labor-intensive genetic screening method. Direct sequencing is typically the most time-consuming step in the identification of point mutations, even with the advent of automated sequencing methods. Further, even DNA sequencing may not give a clear indication of a point mutation in some cases, for example when an individual is heterozygous for that allele.

Non-specific background signals are often present in sequencing reactions, appearing as coincident bands on the sequencing ladder in manual sequencing methods using radioisotopes (Cheng and Haas, 1992), or appearing as non-specific fluorescent peaks in automated sequencing reactions. The main causes of this type of background are premature termination during the extension reaction and non-specific priming. Due to these limitations, and to the time and expense involved in sequencing large regions of DNA, direct sequencing is more practical as a tool to identify the specific nucleotide alterations in samples known to contain mutations, rather than as a primary screening method to assess large regions of the genome. Therefore, preliminary screening methods are needed to identify samples that contain mutations, and avoid the unnecessary labor, expense, and time needed for sequencing samples which do not contain mutations.

The most common screening method currently in use is SSCP. This method involves amplification of target regions, usually less than 300 bp long, which are denatured and separated on thin, native polyacrylamide gels. Point mutations are detected as mobility differences between wild-type controls and experimental samples. One drawback of SSCP is the requirement for radiolabeled material for analysis, due to the small mass amounts of the double-stranded DNA samples that must be used to prevent reannealing of the complementary strands after denaturation.

Another disadvantage of SSCP is that the gels typically require long running periods (6-18 hours or longer) at high voltages, usually in a cold room with recirculation of the running buffer. These electrophoresis parameters are awkward, labor-intensive, hazardous, and require the use of expensive and specialized equipment. In addition, no single, optimal electrophoresis condition or gel composition for detection of mutations by SSCP, has yet been discovered. Therefore, each sample is typically assessed on multiple gels (for example, with and without 10% glycerol, or at room temperature and 4°C). Further, as the size of the target region assessed by SSCP increases, the detection rate decreases. For example, in one study, the detection rate decreased to 57% in 307 bp

targets (Sarker *et al.*, 1992). Therefore, SSCP is not effective for screening large regions of the genome in a single step.

Another method of screening for point mutations is based on RNase cleavage of base pair mismatches in RNA/DNA and RNA/RNA heteroduplexes. As used herein, the term "mismatch" is defined as a region of one or more unpaired or mispaired nucleotides in a double-stranded RNA/RNA, RNA/DNA or DNA/DNA molecule. This definition thus includes mismatches due to insertion/deletion mutations, as well as single and multiple base point mutations. U.S. Patent No. 4,946,773 describes an RNase A mismatch cleavage assay that involves annealing single-stranded DNA or RNA test samples to an RNA probe, and subsequent treatment of the nucleic acid duplexes with RNase A. After the RNase cleavage reaction, the RNase is inactivated by proteolytic digestion and organic extraction, and the cleavage products are denatured by heating and analyzed by electrophoresis on denaturing polyacrylamide gels. For the detection of mismatches, the single-stranded products of the RNase A treatment, electrophoretically separated according to size, are compared to similarly treated control duplexes. Samples containing smaller fragments (cleavage products) not seen in the control duplex are scored as +.

Currently available RNase mismatch cleavage assays, including those performed according to U.S. Patent No. 4,946,773, require the use of radiolabeled RNA probes. The use of radiolabeled probes has many drawbacks, including the expense of the isotope, the time delay required for probe synthesis and film exposure, and, particularly, the hazard radioisotopes present to workers during their synthesis, purification, and use at close range in the assay. The problems and costs associated with disposal of radioactive waste are also serious and well-documented.

Further drawbacks of RNase mismatch cleavage assays in their present form, include the fact that only about one half to two thirds of point mutations are detected (Myers *et al.*, 1985; Theophillus *et al.*, 1989; Grompe, 1993). In light of these limitations, RNase mismatch cleavage assays have largely fallen into disuse.

Myers and Maniatis in U.S. Patent No. 4,946,773 describe the detection of base pair mismatches using RNase A. Non-specific cleavage seen with RNase A has been a significant problem (Theophillus *et al.*, 1989; Maniatis *et al.*, U.S. Patent No. 4,946,773). RNase A levels needed for optimum cleavage of mismatches are so high as to cause significant non-specific cleavage in the no-mismatch controls. Non-specific cleavage by RNase I has also been reported to be a problem when using this enzyme for mismatch detection (Ekenburg and Hudson, 1994).

Subsequent to the issue of the Myers and Maniatis patent, the *E.coli* enzyme, RNase I, was tested by the inventor for use in mismatch assays. Because it has broader cleavage specificity than RNase A, RNase I would be a desirable enzyme to employ in the detection of base pair mismatches if components can be found to decrease the extent of non-specific cleavage and increase the frequency of cleavage of mismatches.

Unfortunately, the use of RNase I has proven difficult. The use of RNase I for mismatch detection is described in literature from Promega Biotech (Ekenberg and Hudson, 1994). Promega markets a kit containing RNase I that is shown in their literature to cleave three out of four known mismatches, provided the enzyme level is sufficiently high. One drawback of the Promega kit is that a very large amount of the RNase I enzyme must be used. For detection of single base mismatches, the Promega Technical Manual recommends using approximately 100 times more RNase I than is normally used in the standard RNase protection assay. The amount of RNase I required to cleave mismatches results in partial degradation of the base paired control duplex. (Promega Technical Manual, 1994). Non-specific cleavage of the double-stranded duplex makes interpretation of the data more difficult and error-prone.

A technique that can reliably detect point mutations in long and short target regions of nucleic acids in a safe, non-labor intensive, cost effective way, would represent a significant advantage. A technique that avoids the necessity for using radioactive labels would be particularly advantageous. The development of such a

technique would likely increase the utility of mutation detection and its application in numerous clinical and research settings. Finally, the development of a protocol that allows use of RNase I would be advantageous.

SUMMARY OF THE INVENTION

The present invention seeks to overcome the drawbacks inherent in the prior art by providing methods and compositions to improve the detection of mutations using RNase protection assays. The invention concerns RNase mismatch cleavage assays that use an alternative substrate, a different method of product analysis and/or new components in the RNase digestion buffer. The present invention improves on the present mismatch cleavage methods by providing methods that are faster and less labor intensive, that do not require the use of radioisotopes and, most importantly, that results in a significant improvement in mutation detection rate.

The target (substrate) for use in this invention will preferably be an RNA-RNA target. These aspects are therefore in contrast to the DNA-RNA target most commonly employed in the art. Analysis of RNase cleavage products for mutation detection has, until now, exclusively used denaturing conditions to render the cleavage products single-stranded. In contrast, this invention utilizes a non-denaturing analysis to separate double-stranded nucleic acid products. This allows the subfragments to be visualized by staining with DNA intercalating dyes, such as ethidium bromide, and by-passes the need for RNase inactivation prior to analysis of cleavage products.

As the RNase cleavage products remain double-stranded throughout the analysis, and therefore resistant to degradation by single-strand specific nucleases, inactivation of the RNase prior to analysis of the products is not required. This results in a major reduction in hands-on time and turnaround time needed to perform the assay. Also, the effects of non-specific cleavage are minimized when the cleavage products remain double-

stranded during analysis, allowing significantly longer target regions to be screened in a single step compared to SSCP.

In a preferred manner of using the invention, large mass amounts of RNA are generated by *in vitro* transcription of normal (wild type control) and test templates generated by PCR™. However, this is not the only contemplated method. For example, RNA could simply be isolated from the cells to be analyzed. The PCR™ technique is the subject of U.S. Patent No. 4,683,202, and is well known to those of skill in the art. The advent of PCR™ makes it straightforward to generate templates for *in vitro* transcription of test and control target sequences into RNA. The incorporation of opposable phage promoter sequences (T7, SP6, T3) into amplified PCR™ products to permit their transcription by phage polymerases is described.

The present invention discloses a mismatch cleavage assay in which RNA transcripts of test sample and controls are produced by *in vitro* transcription of PCR™ products containing opposable T7 and SP6 phage promoter sequences. No radioisotopes are required in the disclosed method, termed non-isotopic RNase cleavage assay ("NIRCA™"), since the dual amplification steps of PCR™ and *in vitro* transcription yield large amounts (several micrograms) of target substrate, which permits the cleavage products to be visualized directly under UV light, when stained with ethidium bromide.

In the invention, hybridization of complementary wildtype and test transcripts creates double-stranded nucleic acid molecules, preferably RNA-RNA target molecules, which may be substrates for RNase cleavage if they contain mismatched residues. Cleavage of mismatched RNA at nucleotide residues on both strands of the duplex results in subfragment(s) which can be distinguished by electrophoresis on native (non-denaturing) gels. This is also an unexpected feature of the present invention, as RNase A is only capable of cleaving pyrimidine bases and would not be expected to cleave strands with mismatched purines (Griffin and Griffin, 1994).

The present invention thus provides improved methods for detecting a base pair mismatch, that involve generating a double-stranded nucleic acid duplex, subjecting the duplex to digestion, and separating the cleavage products under conditions that allow them to remain double-stranded.

Inventive methods of detecting a base pair mismatch in a nucleic acid sample, wherein the nucleic acid sample may be prepared in a double-stranded form, generally comprise the steps of: (a) preparing or otherwise obtaining a single stranded nucleic acid test sample to be analyzed; (b) contacting the said test sample with a single stranded nucleic acid probe under conditions effective to form a test nucleic acid duplex; (c) treating the test duplex with a ribonuclease composition capable of cleaving double-stranded nucleic acid molecules containing base pair mismatches under conditions effective to allow the formation of cleavage products; and (d) separating the cleavage products so formed under conditions that allow the cleavage products to remain double-stranded.

Naturally, the single stranded nucleic acid probe used will be a probe that has a substantially complementary sequence to the nucleic acid being analyzed. In one preferred embodiment, it will have exactly the same sequence as the wild-type form of the nucleic acid segment or gene in question. In another preferred embodiment, it is contemplated that the single stranded RNA probe will have a sequence that has exactly the same sequence as the sequence of the commonly-observed mutation that one suspects may be present in the sample of unknown sequence.

The RNA test sample, the RNA control sample, or both, may be prepared by transcription of a recombinant plasmid or by transcription of a PCR™ product. In a preferred embodiment, the target for the *in vitro* transcription reaction will be one that contains opposable promoters, *i.e.*, promoters that interact with different or the same RNA polymerase enzymes (SP6, T7 and T3 RNA polymerases) in order to effect translation. Examples of preferred promoters are the SP6, T7 and T3 promoters, any

combination of which may be employed. In a form of the method that involves simultaneous co-transcription of both strands of the template, the opposable promoters used may be the same, allowing transcription by a single RNA polymerase.

The cleavage products produced in these assay methods are preferably separated using a non-denaturing system, such as by using a non-denaturing agarose gel, a non-denaturing polyacrylamide gel, non-denaturing HPLC, capillary electrophoresis or any other non-denaturing method.

The use of non-denaturing gel electrophoresis is currently preferred. In a preferred method, the cleavage products are admixed with a high ionic strength loading solution to form a loading sample prior to separation by non-denaturing gel electrophoresis. The present invention provides novel loading solutions for this purpose that comprise a salt in a concentration sufficient to provide a final salt concentration in each loading sample of at least about 0.5 M. The salt may be a tetramethyl alkyl salt, such as tetramethylammonium chloride, or a more common salt, such as NaCl.

For detection, the cleavage products may be contacted with an agent, such as ethidium bromide, that causes them to fluoresce. The agent may be incorporated into the non-denaturing gel or added to the sample before gel separation. Alternatively, the cleavage products may be analyzed by silver staining. Automated devices may also be used to analyze the cleavage products.

The single stranded RNA probe may have a sequence of the wild type gene and thus form a duplex with said control sample that does not contain a mismatch. It may also have a sequence of the mutant gene and thus form a duplex with said control sample that contains a mismatch. The single stranded RNA test sample is obtained, for example, from a patient suspected of having a disease associated with a genetic mutation, such as cancer. A preferred embodiment of this invention employs a *p53* gene.

Sensitivity (*i.e.*, the ability to detect 100% of all mutations) and specificity (lack of false positives) are important requirements for widespread acceptance of any new method for genetic screening. Even though the NIRCA™ method is a considerable improvement over past systems, not all point mutations are detected by NIRCA™. A reduced sensitivity of NIRCA™, relative to more laborious and expensive screening methods (*e.g.*, SSCP, DGGE, direct sequencing) is its major disadvantage. Any method to improve the sensitivity of the NIRCA™ assay would increase its utility for mutation detection and genetic analysis in many areas of basic and applied research, as well as in clinical/diagnostic settings.

In view of the above, the inventor has developed novel reagents for use in NIRCA™ assays that improve sensitivity. The invention concerns compositions for performing the RNase digestion step of the assay, which differ substantially from any which have been previously described. The invention reduces non-specific cleavage and the amount of enzyme needed for cleavage of mismatches for at least some RNases, and results in the cleavage of many base pair mismatches which are completely resistant to cleavage under previously reported conditions. The invention also results in more complete cleavage of many mismatches which were only partially or marginally cleaved under known conditions. The reduction in non-specific cleavage and increase in frequency and extent of mismatch cleavage result in a significant improvement in the sensitivity and specificity of the RNase cleavage-based genetic screening assay. In addition, the invention appears to increase the activity of at least some RNases on single-stranded substrates, that is, the single-stranded substrates will be degraded at lower enzyme concentrations than would otherwise be required.

The present invention uses a reaction mixture comprising a) an RNase enzyme; b) a nucleic acid intercalating agent; and c) an RNase mismatch cleavage activity enhancing agent comprising a protein mixture, a divalent cation, or both.

The present invention also provides methods and compositions which improve the frequency and extent of cleavage of base pair mismatches in double stranded nucleic acid targets. The present invention provides a novel reaction mixture which increases the ability of RNases, particularly RNase I and RNase T1, to specifically cleave base pair mismatches in duplex nucleic acids. Despite the favorable characteristics of RNase I in that it has broader cleavage specificity in single stranded RNA targets, the fact that many mismatches ere uncleaved has effectively prevented the use of RNase I for detecting base pair mismatches. Of even greater concern is the fact that even when the recommended high levels of RNase I are used, many single-base mismatches are not cleaved using the reaction mixture in the Promega Technical Manual (FIG. 4).

In a preferred embodiment, the present invention comprises ribonucleases such as RNase I, RNase T1, and RNase A used alone or in combination, in a reaction mixture comprising (a) an intercalating agent; (b) certain divalent cations and/or a digested protein mixture.

Another embodiment of the present invention is a method for identifying a mutation in a gene; comprising the steps of: obtaining a single stranded RNA test sample from said gene and a single stranded RNA non-mutant control sample having the wild type sequence of said gene; contacting said test RNA sample and said non-mutant control RNA sample with a single stranded RNA probe, thereby forming a test RNA duplex and a control RNA duplex; treating said test RNA duplex and said control RNA duplex with a ribonuclease composition capable of cleaving double-stranded RNA molecules containing base pair mismatches, under conditions effective to allow the formation of cleavage products, said ribonuclease composition comprising: (1) an RNase enzyme; (2) a nucleic acid intercalating agent; and (3) an RNase mismatch cleavage activity enhancing agent comprising a digested protein mixture, a divalent cation, or both; separating said cleavage products under conditions that allow the cleavage products to remain double-stranded; and comparing the separated cleavage products from said test RNA duplex and said control RNA duplex, wherein a difference in the size of the cleavage products is indicative

of the presence of a mutation in said gene. A kit for conducting such an assay is also contemplated by the invention.

The present invention represents a major technical advance by identifying reaction conditions which result in significant improvement in specific mismatch cleavage. The invention is also expected to improve detection of mutations using the methods of the conventional RNase cleavage assay (Myers *et al.*, 1985; Maniatis *et al.*, U.S. Patent No. 4,946,773, 1990), where cleavage products are denatured and analyzed as single strands. This is because any agent which improves cleavage of both mismatched strands must necessarily improve cleavage of the individual strands.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Detection of Mutations in DNA targets using radiolabeled probes analyzed on denaturing polyacrylamide gel using the Conventional RNase Protection Assay of the prior art. Note the presence of subfragments in the samples scored as positive for mutations in lanes 1, 6, and 8 (probed with Sense strand probe) and in lanes 5, 6, and 8 (same targets probed with Antisense probes). Also note the presence of nonspecific subfragments present in all lanes, which are due to cleavage by RNase A at hypersensitive sites. Note that the sizes and number of the nonspecific fragment differ depending on whether the Sense or the Antisense probe was used. The no-mismatch controls are in lane 3 in both panels.

FIG. 2. Detection of mutations in 969 bp targets using the Non-isotopic RNase Cleavage Assay of the present invention. Lanes 1-14 show cleavage products from

substrates made by hybridizing patient Sense transcripts with wildtype Antisense transcripts; Lanes 15-28 show cleavage products from substrates containing patient Antisense and wildtype Sense transcripts. The no-mismatch control substrates treated with RNase are in lanes 5 and 19. The last lane (mw) has pUC19/Sau 3A restriction fragments of the indicated sizes.

FIG. 3. Analysis of double-stranded RNA cleavage products on native agarose gels in either standard or high ionic strength gel loading solution using the Non-isotopic RNase Cleavage Assay of the present invention. Note that the bands in the left-hand panel (standard ionic strength gel loading solution) are generally broader, less distinct, and more poorly resolved than those in the right-hand panel (high ionic strength gel loading solution). Results similar to those in the right-hand panel were seen when 3M NaCl (sodium chloride) was used instead of 3M TMAC.

FIG. 4. Cleavage of mismatches using prior art and current invention compositions for RNase I digestion buffer.

FIG. 5. Cleavage of mismatches using RNase T1.

FIG. 6. Cleavage of mismatches using digestion buffer with calcium instead of tryptone.

FIG. 7. Cleavage of mismatches in the presence of acridine orange.

FIG. 8. Effects of tryptone, calcium and the metal chelators EDTA and EGTA on mutation cleavage. FIG. 8 is composed of five panels: FIG. 8A, FIG. 8B, FIG. 8C, FIG. 8D, and FIG. 8E.

FIG. 9. Cleavage of mismatches in *p53* targets by RNase A, RNase I, and RNase T1. FIG. 9 is composed of three panels: FIG. 9A, FIG. 9B, and FIG. 9C.

FIG. 10. Cleavage of mismatches in a large panel of homozygous and heterozygous samples with Factor IX mutations. FIG. 10 is composed of two panels: FIG. 10A and FIG. 10B.

DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS AND EXAMPLES

I. Detailed Description

The present invention arises from the discovery of improved methods and compositions of mismatch detection in the RNase mismatch cleavage assay. The improvement lies in the use of intercalating agents, divalent metal ions and elements of a protein mixture in the reaction mixtures. Through the use of these improved methods and compositions, marked improvement is seen. The compositions are effective for specific cleavage of base pair mismatches in double-stranded nucleic acid, and especially in double-stranded RNA targets.

A. Detailed Description of RNase Mismatch Cleavage and Protection Assay For Use In Detecting Mismatches

The RNase protection assay as first described by Melton *et al.* (1984) was used to detect and map the ends of specific mRNA targets in solution. The assay relies on being able to easily generate high specific activity radiolabeled RNA probes complementary to the mRNA of interest by *in vitro* transcription. Originally, the templates for *in vitro* transcription were recombinant plasmids containing bacteriophage promoters. The probes are mixed with total cellular RNA samples to permit hybridization to their complementary targets, then the mixture is treated with RNase to degrade excess unhybridized probe. Also, as originally intended, the RNase used is specific for single-stranded RNA, so that hybridized double-stranded probe is protected from degradation. After inactivation and removal of the RNase, the protected probe (which is proportional in

amount to the amount of target mRNA that was present) is recovered and analyzed on a polyacrylamide gel.

The RNase Protection assay was adapted for detection of single base mutations by Myers and Maniatis (1985) and by Winter and Perucho (1985). In this type of RNase A mismatch cleavage assay, radiolabeled RNA probes transcribed *in vitro* from wildtype sequences, are hybridized to complementary target regions derived from test samples. The test target generally comprises DNA (either genomic DNA or DNA amplified by cloning in plasmids or by PCR™), although RNA targets (endogenous mRNA) have occasionally been used (Gibbs and Caskey, 1987; Winter *et al.*, 1985). If single nucleotide (or greater) sequence differences occur between the hybridized probe and target, the resulting disruption in Watson-Crick hydrogen bonding at that position ("mismatch") can be recognized and cleaved in some cases by single-strand specific ribonuclease. To date, RNase A has been used almost exclusively for cleavage of single-base mismatches, although RNase I has recently been shown as useful also for mismatch cleavage. There are recent descriptions of using the MutS protein and other DNA-repair enzymes for detection of single-base mismatches (Ellis *et al.*, 1994; Lishanski *et al.*, 1994).

By hybridizing each strand of the wildtype probe in RNase cleavage mismatch assays separately to the complementary Sense and Antisense strands of the test target, two different complementary mismatches (for example, A-C and G-U or G-T) and therefore two chances for detecting each mutation by separate cleavage events, was provided. Myers *et al.* (1985) used the RNase A cleavage assay to screen 615 bp regions of the human β globin gene contained in recombinant plasmid targets. By probing with both strands, they were able to detect most, but not all, of the β-globin mutations in their model system. The collection of mutants included examples of all the 12 possible types of mismatches between RNA and DNA: rA/dA, rC/dC, rU/dC, rC/dA, rC/dT, rU/dG, rG/dA, rG/dG, rU/dG, rA/dC, rG/dT, and rA/dG.

Myers *et. al.* (1985) showed that certain types of mismatch were more frequently and more completely cleaved by RNase A than others. For example, the rC/dA, rC/dC, and rC/dT mismatches were cleaved in all cases, while the rG/dA mismatch was only cleaved in 13% of the cases tested and the rG/dT mismatch was almost completely resistant to cleavage. In general, the complement of a difficult-to-detect mismatch was much easier to detect. For example, the refractory rG/dT mismatch generated by probing a G to A mutant target with a wildtype sense-strand probe, is complemented by the easily cleaved rC/dA mismatch generated by probing the mutant target with the wildtype antisense strand. By probing both target strands, Myers and Maniatis (1986) estimated that at least 50% of all single-base mutations would be detected by the RNase A cleavage assay. These authors stated that approximately one-third of all possible types of single-base substitutions would be detected by using a single probe for just one strand of the target DNA (Myers *et al.*, 1985).

It is generally accepted that RNase A is specific for cleavage on the 3′ side of pyrimidine residues in single-stranded RNA, and does not cleave at unpaired purines (A or G) (Richard and Wyckoff, 1971; Adams *et al.*, 1992). In general, Myers *et al.* (1985) found little or no cleavage of purine-purine mismatches in RNA/DNA duplexes. However, Myers *et al.* noticed that, "surprisingly", RNA strands containing purine mismatches were cleaved in some cases. They attributed such cleavage events to cleavage at nearby susceptible pyrimidine residues, which were thought to be destabilized by proximity to the mismatch.

The current teaching in the art is that mismatched purines are not cleaved by RNase A (in PCR™ Technology, Current Innovations, eds. Griffin and Griffin, page 115 (1994)). Since most mismatches contain at least one purine residue, and as RNase A is not believed to be effective against purines, the combined teaching of the art appears to be that efficient double strand cleavage of a mismatched RNA-RNA duplex would not be possible.

For the foregoing and other reasons, prior to the present invention, products of RNase cleavage assays have been analyzed on denaturing polyacrylamide gels. Various other factors also dictated that denaturing gel analysis be used in the protection assay methods as originally described. For example, RNase cleavage assays have traditionally used DNA targets, in which case double-strand cleavage of the mismatched RNA-DNA heteroduplex by RNase A would not have occurred, since RNase A does not cleave DNA. The product of RNase A mismatch cleavage of an RNA probe/DNA target heteroduplex is a double-stranded molecule with a nick in the phosphodiester backbone of the RNA strand, and the mobility of such a molecule on a non-denaturing (native) gel would not be altered compared to the uncleaved molecule. The presence of a mutation in a DNA target would therefore not be detectable by RNase cleavage unless analyzed on a denaturing gel.

Even in RNase cleavage assays using RNA-RNA targets, the generally held belief that RNase A does not effect significant cleavage at purine residues in single-stranded RNA, would lead to the conclusion that an analysis of mismatch cleavage products in RNA-RNA duplexes would only be feasible using denaturing gels. Also, denaturing conditions are used to assure that the mobility of the subfragments generated by RNase cleavage will be proportional to their size, so that the relative positions of different fragments in the gel will be an indication of the relative positions of the mutations. Additionally, mobility differences due to secondary structure, rather than size, might cause different fragments to co-migrate and perhaps obscure data.

In the typical RNase cleavage assays, the need to run separating gels under denaturing conditions means that the RNase must be inactivated before the cleavage products can be analyzed. Otherwise, the RNA probe would be completely degraded by RNase upon being separated from its target DNA (or RNA). RNase inactivation in these prior art methods required that the reaction be treated with protease (usually Proteinase K, often in the presence of SDS) to degrade the RNase. This reaction is generally followed in the prior techniques by an organic extraction with a phenol/chloroform

solution to remove proteins and residual RNase activity. The organic extraction is then followed by concentration and recovery of the cleavage products by alcohol precipitation (Myers *et al.*, 1985; Winter *et al.*, 1985; Theophilus *et al.*, 1989).

RNase inactivation as currently practiced is therefore a labor-intensive, multistep process. Phenol/chloroform extraction is especially problematical due to the requirement for close contact of laboratory personnel with caustic, radioactive and/or toxic chemicals, and the subsequent physical separation and removal of an upper liquid phase from a lower liquid phase containing contaminating material. Such manipulations are not easily accomplished by automated methods (*e.g.*, robotic arms). The alcohol precipitation step involves addition of ethanol or isopropanol to all samples, thorough mixing, storage at -20°C for at least 15 minutes, and centrifugation of samples for about 15 minutes in a microcentrifuge. The supernatant fluid must then be removed from each sample and the remaining pellet of nucleic acid thoroughly resuspended in a small volume of denaturing loading buffer (usually 80% formamide and tracking dye) by vigorous vortexing, heated for about 3 minutes at 95°C, and then loaded on a thin polyacrylamide gel. These steps also require close contact of personnel with hazardous material (radiolabeled RNA) and do not lend themselves to automation. The RNase inactivation and sample recovery steps add several hours of mostly hands-on time to the RNase mismatch cleavage assay as practiced prior to this invention.

Despite the teaching of the prior art to the contrary, the present inventors have made surprising discoveries that have allowed the development of an improved RNase mismatch assay that employs analysis of the RNase cleavage products on native gels. This development is unexpected as even in a previous example where RNase mismatch cleavage products were analyzed on agarose gels, rather than polyacrylamide gels, the sample was still dissolved in denaturing buffer, heat denatured, and run on a denaturing (formaldehyde) agarose gel (Genovese *et al.*, 1989).

In preferred embodiments of this invention, RNase A is used to cleave strands containing both mismatched purines and pyrimidines in a double-stranded RNA molecule, to form cleaved double-stranded products. This is followed by separation of the double-stranded RNA cleavage products under non-denaturing conditions. This is an important step that now permits direct analysis of cleavage products without RNase inactivation and together with the two step amplification procedure permits direct visualization of reaction products without the use of radioisotopes.

The present inventor applied certain existing technologies, such as *in vitro* transcription of PCR™ products containing opposable phage promoters and non-denaturing electrophoresis, in the design of the improved RNase cleavage assays described herein. However, the combination of techniques employed by the inventors has not been suggested previously due, in part, to certain rigidly-held beliefs concerning RNase substrate bases. Presently, PCR™ and *in vitro* transcription are used to generate large mass amounts of RNA to serve as both wildtype probes and test targets for detecting mutations using the RNase cleavage assay. The new RNase cleavage assay is designed to take advantage of the observation that cleavage frequently occurs on both strands (notwithstanding current teaching to the contrary), and also to take advantage of a PCR™ strategy for the preparation of double stranded RNA substrates. Finally, non-denaturing conditions are used for separating the end products to analyze for cleaved RNA duplexes.

The invention thus provides improved methods and compositions for analyzing the RNase cleavage products rapidly, with inexpensive reagents and apparatus, without the use of radioisotopes. A further advantage of the present invention is that these new methods allow significantly larger regions of genetic sequence to be analyzed in a single step, compared to other methods in common use. The primary modification in the assay that results in these improvements is the fact that the products of the nuclease cleavage remain double-stranded during the analysis. This is because the samples are analyzed on native gels instead of the denaturing gels used in the prior art. Maintenance of the

double-stranded form of the cleavage products throughout the analysis means that the products are resistant to RNase degradation, and the labor intensive, time consuming steps associated with nuclease inactivation are eliminated.

An additional major benefit of keeping the cleavage products double-stranded during the analysis is that the effects of non-specific background cleavage, long known to be a problem in mismatch cleavage assays (Myers *et al.*, 1985; Theophilus *et al.*, 1989), are minimized. This is because non-specific cleavage in many cases generates single-stranded nicks under the conditions used for mismatch cleavage, rather than the double-stranded cleavage events that would be required to generate detectable background fragments on a non-denaturing gel.

Native agarose gel electrophoresis has been used for many years in the analysis of double-stranded DNA, for example, restriction endonuclease cleaved or uncleaved plasmid and phage DNA, PCR™ products, chromosomal DNA, *etc.* The DNA fragments are typically in the size range of 50 bp - 50,000 bp, more commonly in the 300 bp - 10,000 bp range. The DNA is generally visualized by staining with the intercalating dye, ethidium bromide, which can be added to the sample itself, and/or to the agarose gel, and/or to the buffer.

Ethidium has the property of binding to nucleic acids in a planar array between successive nucleotides ("intercalation"). Ethidium bound to nucleic acid absorbs light optimally in the ultraviolet (uv) range (~260 nm) and fluoresces (emits visible light) in the orange-red range (590 nm) (Sambrook, 1989). The sensitivity limit for detection of ethidium-stained double-stranded DNA with the bare eye is ~ 10 ng when viewed on a "transilluminator", a simple inexpensive apparatus which includes a panel of uv light bulbs mounted beneath a uv-transparent flat glass surface. Agarose gels containing electrophoretically separated nucleic acids are placed onto the transilluminator and assessed by inspection from above. The viewer must always wear uv-protective goggles and a face shield to protect against uv-irradiation of eyes and skin. Alternatively, the UV

stained products may be detected on a computer screen using instruments designed for visualizing and documenting ethidium stained nucleic acid fragments in gels.

For separation of nucleic acid fragments, the gels are submerged in an electrolyte solution ("running buffer") such as Tris-boric acid-EDTA, and samples containing the nucleic acid in a dense solution are placed into the bottom of wells cast in the gel bed. The fragments are distinguished by mobility differences under the influence of an electric field applied across the gel. For double-stranded nucleic acid molecules, which take the form of a stiff rod, the distance migrated from the well is inversely proportional to the log of the molecular weight of the fragment; the shorter the double-stranded DNA molecule, the faster it migrates, and the greater is its distance from the origin after electrophoresis for a set time.

The basic technique of analyzing ethidium-stained double-stranded DNA molecules by electrophoresis on native agarose gels has been used in molecular biology for many years, however, it has not been used in the context of RNase cleavage product analysis. Neither has non-denaturing agarose gel electrophoresis been widely applied for analysis of single-stranded RNA. One reason for this is that, in contrast to double-stranded nucleic acid molecules, the affinity of single-stranded nucleic acid for ethidium is relatively low, and the fluorescent yield is comparatively poor (Sambrook, 1989). The fluorescent detection of ethidium stained single-stranded RNA on agarose gels therefore requires a greater mass amount of RNA than is needed for similar detection of double-stranded nucleic acid.

A second reason that single-stranded RNA molecules are not widely analyzed on native agarose gels, for example, as part of the Northern blot procedure, relates to the electrophoretic mobility of single-stranded RNA molecules under non-denaturing conditions. Single-stranded molecules migrate in an electric field at a rate that depends on both their size and their nucleotide sequence. The observed mobility differences between same-sized molecules with slightly different sequences, even a single nucleotide, form the basis for

one widely used method for detecting point mutations, SSCP (single-stranded conformation polymorphism) analysis. In situations where it is desirable for the mobility of the single-stranded nucleic acid to depend only on its size (for example, in Northern blotting, and in ribonuclease protection analysis of mRNA) and not its sequence, the sample is electrophoretically separated on denaturing, as opposed to native gels.

Denaturing agents, such as formaldehyde, methyl mercury hydroxide, urea, and glyoxal, eliminate intrastrand and interstrand interactions that cause secondary structure of the nucleic acid, for example base pairing between nucleotides. In general, denaturing conditions are defined as those that abolish or prevent Watson-Crick base pairing between complementary strands. Fractionation of complex mixtures of mRNA on denaturing agarose gels followed by hybridization to specific complementary radiolabeled probes ("Northern blot" analysis) permits identification of specific target mRNAs and their characterization according to size. Similarly, the products of RNase digestion of radiolabeled probes hybridized to complex RNA mixtures are detected and analyzed on denaturing polyacrylamide gels (the "RNase Protection" assay). The choice between polyacrylamide and agarose for the gel matrix depends on the size of the fragments and on the degree of resolution required, with polyacrylamide typically being used for high resolution of small (<1000 bases) molecules.

Since the RNase protection assays currently available in the art involve the separation and analysis of single-stranded products, there is a lack of suggestion in the art that non-denaturing gels, such as native agarose gels, could be employed as part of the technique. This, in turn, leads to the detection limitation that means ethidium bromide staining is unlikely to be effective. Thus, there is little scope for using agents other than radiolabelled or silver stained compounds in the analysis.

The invention provides improved methods that will be readily adapted for use in routine screening protocols, for example, as may be employed in a clinical setting. For such routine analysis of many samples, it is particularly important to avoid the use of

radioactive agents. The methods of the present invention provide for the detection of point mutations, as may be indicative of genes involved in the etiology of cancer, or of individuals predisposed to develop cancer, or important in the prognosis of cancer patients, using a nuclease cleavage assay that does not require the use of nucleic acid probes labeled with radioisotopes or other extrinsic labels (*e.g.*, biotin, fluorescein, digoxigenin). These methods are also less labor intensive, less time consuming and less expensive, allowing for high volume screening.

B. Preferred Enzymes

There is a large literature describing different ribonucleases, a number of which are commercially available. Many enzyme may be expected to exhibit single-base mismatch cleavage properties and would be included in the scope of the present invention. Presently, depending on reaction conditions, RNase A and RNase I are preferred enzymes.

RNase A is found in many organs in vertebrates, but is present in concentrated form in the pancreas. The RNase A used for mismatch cleavage in the present invention may be isolated from bovine pancreas or from pancreas of other mammalian species, or may be one of a large family of related RNases isolated from other mammalian organs or sources (for example, brain or serum), or may be a genetically altered RNase A analog, such as those described by Raines and del Cardayre, U.S. Patent No. 5,389,537. The RNase A may be present at a concentration of about 0.1-0.5 micrograms/ml. This concentration is about 400-fold lower than the amount historically used in the RNase protection assay. Historically, preferred concentrations of RNase A to be used for mismatch cleavage have been given in units of mass, rather than activity (Myers *et al.*, 1985; Maniatis *et al.*, US Patent No. 4,946,773). The mass amount of RNase A used may be converted to units of activity defined as follows (Ambion unit definition): one unit of RNase A is the amount needed to give an increase in absorbance at 286 nm of

0.0146 units per minute, when incubated with 1 mM cyclic CMP substrate in a volume of 1 ml.

RNase A-like enzymes from a large number of mammalian species (sheep, pig, rat, horse, kangaroo, human, and others) have been isolated and characterized. The many scattered amino acid substitutions found in the different RNases suggest that the enzymes may have different properties and cleavage specificities.

In certain embodiments, it is contemplated that commercially available RNase A will be preferred for use with the present invention. RNase A may be easily obtained from commercial sources, for example, from Sigma Chemical Company (Catalog No. R-5125). However, it is well known in the art that such commercially available "RNase A" is, in fact, often a combination of RNase A and RNase B, which represent differently glycosylated forms of the enzyme, and thus the use of an RNase A and B mixture is contemplated. Alternatively, RNase A which is essentially free of RNase B, for example, that which is available from Sigma (Catalog No. R-5250) may be used. RNase B (Sigma Catalog No. R-5875) is also encompassed in the scope of the present invention. The RNase A preparation may generally be dissolved in distilled water at a concentration of about 2 mg/ml, placed at 100°C for 10 minutes, slowly cooled to room temperature and then stored at -20°C or 4°C for upwards of one year.

In addition, RNase A mutants may be generated by *in vitro* mutagenesis and over-expression in *E. coli*. Mutagenesis can be accomplished by standard methods such as site directed mutagenesis. Some existing mutants which are presently available appear to have a much increased ability to cleave at purine residues (Raines, 1991).

RNase I is found in the periplasmic space of *E. coli*. It is likely that enzymes similar to RNase I will be found in other bacterial species localized in the periplasmic space. The enzyme may be RNase I isolated from *E. coli*, or may be another *E. coli* enzyme with similar properties and substrate specificity. It may also be isolated from other prokaryotes, eukaryotes, yeast, fungi, or other organisms. Partially purified RNase I

like enzymes may be obtained from Gram negative halophiles, such as *Halobacterium salinarium* and psychrophiles such as *Colwellia psychroerythrus* and others. The enzyme should be present at a concentration to effect optimal specific cleavage of said mismatches, when admixed with the other reaction components. More particularly, RNase I may be present in the reaction mixture at a concentration of from about 0.1 to about 0.5 units per microliter. This concentration is from about 300 to about 60-fold lower than that recommended previously in the art.

One unit of RNase I may be defined as the amount needed to cause 50% degradation of a radiolabeled single-stranded RNA substrate mixed with 2 micrograms of yeast RNA in 30 minutes at 37°C, as determined by trichloroacetic acid precipitation (Ambion unit definition). Alternatively, one unit of RNase I may be defined as the amount required for complete degradation of 2 micrograms of *E. coli* 5S ribosomal RNA in 5 minutes at 37°C (Promega definition). The conversion between Ambion and Promega units of RNase I is thus 12 Ambion units equals approximately one Promega unit, since a Promega unit degrades twice as much substrate (2 micrograms versus 50% of 2 micrograms) in one-sixth the time period (5 minutes versus 30 minutes).

Preferred embodiments of the present invention also include the cleavage of base pair mismatches using RNases isolated from other eukaryotic or prokaryotic sources. These alternate RNases may be used to effect cleavage, especially double-stranded cleavage, of base pair mismatches in duplex nucleic acid targets. Since the methods disclosed herein have been shown to be effective with RNases previously reported to be incapable of mismatch cleavage (*i.e.*, RNase T1) or previously not used or not widely used for mismatch cleavage (*i.e.*, RNase I from *E. coli*, RNase Y1* from yeast), it is contemplated that other RNases and perhaps other endonucleases not heretofore known to be capable of effecting mismatch cleavage may in fact be useful for this purpose. These potentially useful nucleases include, but are not limited to, S1 nuclease, mung bean nuclease, any or all of the RNases in the large family of ribonucleases related to RNase A (see Jermann *et al.*, 1995), RNase T2, the broad specificity RNase from

Aeromonas hydrophila (which is related to RNase I from *E. coli*; Favre *et al.*, 1993), RNase E from *E. coli* (McDowall *et al.*, 1994) and RNases encoded in the *Drosophila melanogaster* genome (Hime *et al.*, 1995). RNases made by random or site-directed mutagenesis of natural RNase genes, for example the RNase A-derived enzymes described by Raines and del Cardayre (U.S. Patent No. 5,389,537 and references therein), are also expected to be useful for mismatch cleavage when used according to the methods set forth in the present disclosure.

The enzyme known as RNase YI*, isolated from yeast, is also effective for cleaving base pair mismatches in duplex RNA when used in conjunction with the ethidium bromide and protein mixture components of the RNase digestion reaction mixture. This enzyme is not commercially available and a strict unit definition of its activity has not been established. It was used at final concentrations ranging from 1:30 to 1:300 dilutions of the stock preparation supplied by Dr. David Kennell. This enzyme is typically used in a concentration necessary to degrade 10 mg poly C to 1-5 mM in 30 min. at 37°C. The enzyme is usable in a buffer of 20 mM TRIS (pH 7.3-7.7), 10 mM Mg^{2+} or 0.1 mM Zn^{2+}, and .5 mM EDTA.

Another preferred embodiment of the present invention encompasses using the fungal RNase, RNase T1, to effect double-strand cleavage of mismatches in duplex RNA targets. The RNase T1 may be isolated from the fungus *Aspergillus orysae* or other *Aspergillus* species or from suitable bacterial host strains overexpressing the cloned fungal gene. The RNase T1 may be present at a concentration of about 100-250 micrograms/ml, corresponding to a unit concentration of about 64,000-160,000 units/ml (where 25 units are defined as the amount of RNase T1 required to degrade 25 micrograms/ml of a GpA dinucleotide substrate to cause an absorbance change of 0.01 A_{260} units in one minute at room temperature, in a reaction mixture containing 50 mM Tris-Cl, pH 7.5, 1 mM EDTA; Ambion definition).

Although RNase T1 was previously reported not to be able to cleave base pair mismatches under various reaction conditions (Myers *et al.*, 1985), the inventor has found that a wide variety of different mismatches are in fact cleaved by this enzyme under the appropriate conditions, to generate double stranded cleavage products from duplex RNA test substrates containing single-base mismatches. In order to achieve optimal mismatch cleavage using RNase T1, high enzyme levels may be used (for example, 64,000-160,000 units/ml in the standard reaction described in Example 8), and the reaction mixture may also contain high concentrations of ethidium bromide or other intercalating agent(s), for example 100 μg/ml ethidium bromide. However, lower RNase T1 concentration and/or lower ethidium bromide concentrations are also effective for cleaving many mismatches. Some mismatches which are partially cleaved using lower RNase T1 and/or lower ethidium concentrations, may be more completely cleaved when the concentrations of these components are raised.

The protein mixture component of certain preferred RNase digestion mixtures, which is required for maximal mismatch cleavage activity by RNase I, does not appear to be required for RNase T1, at least not in the specific mismatches tested thus far. The ability of RNase T1 to effect double-strand cleavage of base pair mismatches in RNA duplexes is surprising, given the reported specificity of RNase T1 for cleavage after guanosine residues only, in single stranded RNA substrates (Worthington enzyme manual, 1993; enzyme 3.1.27.3,described on page 359).

C. Methods of Screening for Genetic Mutations

In scientific detail, a method of screening for genetic mutations of the invention preferably includes the five steps described in the section below. This method utilizes the PCR™ technique as an initial step in the preparation of the RNA. This is a currently preferred means of sample preparation, but is by no means an absolute requirement for practicing the invention.

Step 1. The genetic region to be screened ("target region") is preferably amplified by PCR™ with primers complementary to the sense and antisense strands of the target region, where each primer preferably includes a phage promoter sequence (*e.g.*, T3, T7, SP6) at its 5′ end. The promoters can be identical or different, so that the two strands of the target region can be transcribed by the same or different RNA polymerases (*e.g.*, T7, T3 or SP6 RNA polymerase). Use of the PCR™ to generate transcription templates has been described by Stoflet *et al.*, 1988. The template for the PCR™ can be genomic DNA, cDNA made by RT-PCR™, plasmid or phage or bacterial chromosomal DNA, or mitochondrial DNA, or an existing PCR™ product amplified from any of these sources.

In common practice of the method, the target region may be prepared by a one-step PCR™ or two-step "nested" PCR™, using inner and outer primer pairs. The outer primer pair, which is used in the initial reaction, comprises sequences complementary to the sense and antisense strands of the region to be amplified, chosen for their ability to efficiently amplify singe-copy genomic sequences from the primary source of the test sample, for example clinical material. The first, outer primer pair may be optimized for amplification of a large contiguous region for subsequent screening in smaller subfragments, for instance a 5 kb region may be amplified with the outer primer pair, and screened in 0.5 kb-1 kb increments. The smaller increments are generated with the second, inner primer pair(s). The inner primer pair(s) contain(s) phage promoters added to the 5′ ends of each Sense and each Antisense primer. The inner primer pair(s) are used

to generate the template/ target region which is transcribed with RNA phage polymerase(s) in the next step.

The nested PCR™ strategy increases the specificity of the final product, since spurious products generated by the outer primers are not as a rule amplifiable by the inner primers. The nested PCR™ strategy has been described previously, although not for generating transcription templates to use in the RNase mismatch cleavage assay.

The first step of the method may alternatively be comprised of RT-PCR™ to amplify target sequences expressed as mRNA. In the RT-PCR™ procedure, random-sequence primers or antisense primers specific for the mRNA of interest are used for the reverse-transcriptase catalyzed synthesis of complementary DNA (cDNA). The cDNA is then amplified by using one or more specific primer pairs containing phage promoter sequences (for example T7, SP6, T3) at their 5' ends. As above, the RT-PCR™ may comprise a single step or use a nested PCR™ strategy. The RT-PCR™ is used to amplify all or part of a contiguous coding sequence, expressed as mRNA in clinical or research test samples, into subfragments of about 0.5 kb-1 kb to serve as transcription templates.

The purpose of the first step of the method of this invention ("first step" as used here means one or a series of PCR™ amplifications) is to produce a target region containing opposable phage promoters. The amplified product is typically present in the final PCR™ at a concentration between 0.01 - 0.1 mg/ml, in a reaction volume of 20 μl - 100 μl.

Step 2. In the second step of the method of the invention, the target region containing opposable phage promoters is transcribed into double-stranded RNA to serve as substrate for mismatch detection. The target region is generated by PCR™ in the first step described above. Both strands of the target region are transcribed, either separately or simultaneously, under large-scale synthesis conditions for *in vitro* transcription.

Large scale synthesis conditions are defined here as those having approximately equimolar concentrations of the four ribonucleotides, with each present in the reaction at a concentration of about 0.2-1.5 mM, especially 0.5 mM. Reaction mixtures also contain appropriate buffer, salt, and other components such as spermidine, DTT, and $MgCl_2$, needed for *in vitro* transcription by the phage RNA polymerases (T7, T3, SP6). The reactions also contain about 5-20 units of the RNA phage polymerase(s) corresponding to the phage promoter sequence(s) that were incorporated into the target region during the PCR™ of the first step. The reactions also contain about 1 - 5 μl of the crude PCR™ product of the first step, which comprises the target region being screened.

All components of the *in vitro* transcription reactions, except for the individual targets being screened, may be assembled into a "master mix" which is distributed to the individual reaction vessels, for example wells of a microtiter plate. The master mix may be assembled from stock solutions, for example four stock solutions comprised of 1) a mixture of salts, buffer, spermidine, and reducing agent; 2) a mixture of the four ribonucleotide triphosphates; 3) water; 4) RNA polymerase(s).

For simultaneous transcription of both strands of a target region containing two different opposable promoters, the two corresponding RNA polymerases are both added to the master mix. For simultaneous transcription of a target containing the same promoter at both ends, only a single RNA polymerase is needed. For selective transcription of only one strand of a target containing two different phage promoters, only the single RNA polymerase appropriate for the intended strand to be transcribed, is added to the master mix. For simultaneous transcription of complementary strands, one from the test target region and one from a wildtype control template, both the test template and the wildtype control template (containing the same promoter as that used for the test target, but appended to the opposite end) can be added to the master mix.

The *in vitro* transcription reaction volumes will typically be 5 - 20 μl, especially 10 μl. The reactions are incubated at 37°C for about one hour, and terminated by the

addition of an equal volume of a solution containing formamide, for example 80% formamide/25 mM sodium citrate pH 6.5, followed by heating the reactions for several minutes at about 80 - 100°C in a heat block. The theoretical maximum yield of RNA transcript from a 10 μl reaction containing 0.5 mM concentration of each ribonucleotide is about 6 μg. The typical yield to be expected from such a reaction is about 0.5 μg - 5 μg, especially 2.5-3 μg.

Step 3. Hybridization and RNase digestion of complementary probe and sample RNAs. In this step, Sense and Antisense transcripts from Step 2 are mixed, heated briefly, cooled to room temperature, and treated with a mixture containing ribonuclease(s) capable of specifically cleaving single base mismatches.

In the general procedure, equal volumes of the transcription reactions, for example 2 μl, are mixed such that a transcript derived from a wildtype template (sense or antisense transcript) is combined with a complementary transcript from the test sample. The transcripts may be combined in a 0.65 ml microfuge tube or microtiter plate; these vessels do not need to be specially treated to be RNase-free. The mixture is then heated for about 3 minutes at about 95°C in a heat block and allowed to cool to ambient temperature; during this time, the complementary single strands anneal or hybridize to form a double-stranded RNA molecule. Since the concentrations of the complementary RNA transcripts is high, this process is essentially complete in only a few minutes.

A four-fold volume (for example, 16 μl) of a solution containing RNase (RNase A and/or other RNases such as RNase I, which can specifically cleave single base mismatches), and other components needed for cleavage of unpaired residues (mismatches) in the double-stranded RNA target is then added to an aliquot (for example 4μl) of the hybridized samples and mixed, for example by vortexing or brief gentle manual agitation.

The RNase solution could contain RNase A at a concentration ranging from 0.5 μg–40 μg/ml and tetramethylammonium chloride at a concentration of 0–0.5 M. The RNase solution may also contain other nucleases capable of cleaving at mismatched residues in duplex RNA molecules, for example RNase I in conjunction with the novel digestion buffers of the invention. The reaction is then stored for about 30 minutes at an appropriate temperature, for example 37°C, to allow the nuclease(s) in said reaction to specifically cleave both strands of a duplex RNA containing a base pair mismatch. The optimum time, temperature, and concentration(s) of salt and nuclease(s) are interdependent; for example, specific mismatch cleavage could be maintained by increasing the RNase A concentration and decreasing the time and/ or temperature or increasing the salt concentration during the digestion. Optimization will be a routine matter to those of skill in the art in light of the present disclosure.

As an alternative to mixing aliquots of separate transcription reactions, a single aliquot (for example, 4 μl) of a transcription reaction in which both strands of the DNA template were transcribed simultaneously into RNA, may be treated with RNase as described above. In the situation where the primary test sample comprises genomic DNA containing heterozygous target sequences, then the product of the PCR™ amplification from Step 1 may comprise templates for generating both wildtype and potentially mutant transcripts. In such cases the endogenous wildtype sequences serve to generate Sense and Antisense wildtype probes, circumventing the need to separately transcribe and combine wildtype probes and complementary target transcripts.

Step 4. Analysis of RNase digestion products on native agarose gel (or other non-denaturing gel matrix). In this step, the reactions from Step 3 are loaded onto a gel and double-stranded RNA molecules are separated by electrophoresis under conditions where the complementary strands remain annealed or hybridized (*i.e.*, under non-denaturing conditions).

In the general method, a one-sixth to one-tenth volume of loading solution is added to each reaction after the 30 min. RNase treatment (Step 3). Addition of the loading solution accomplishes several things: 1) it imparts enough density to the sample so that it will sink into the well; 2) by virtue of a tracking dye such as bromophenol blue, it provides a visible indication of the progress of electrophoresis; 3) in a particularly preferred embodiment, the loading solution contains component(s) that cause the nucleic acid molecules to migrate as sharp bands, rather than diffuse areas of stained material.

One possible loading solution composition which meets these requirements is 50% glycerol/ 0.5 M – 3 M tetramethylammonium chloride/ 0.1% bromophenol blue. The appropriate volume of loading solution (for example, 3 μl) is added to the samples and mixed by brief vortexing or manual agitation, or by aspirating the reaction contents up and down several times prior to loading.

The reactions are then loaded directly onto the gel. To load the samples, the contents of each vessel are aspirated up into a pipet tip and then expelled into wells formed in the gel by "combs" (well-forming molds), which are inserted into the molten (un-polymerized) gel. A typical well in an agarose gel has a capacity of about 25-30μl, so that the entire contents of a standard reaction may be loaded. In the general method, the gel may be comprised of 2% agarose in 1 X TBE (90 mM Tris/borate, 2 mM EDTA). The preferred length of the gel is about 4 – 5 cm. At any time prior to loading the samples, ethidium bromide is admixed with the samples to provide a concentration of approximately 0.01-100 μg/ml. The gel is run in 1 X TBE running buffer. After the samples are loaded, a current is applied across the length of the gel to achieve a voltage of about 50-150 volts. The gel may be run at ambient temperature. The exact voltage is not critical, nor is it critical that constant voltage and temperature be maintained.

Recirculation of the running buffer is not required. In the gel system described above, ethidium stained double-stranded RNA molecules migrate toward the positively charged electrode (cathode) at a rate proportional to their length, while the free ethidium

in the sample migrates in the opposite direction, toward the anode. The ethidium which is intercalated between the bases in the RNA remains bound, so that the double stranded RNA fragments may be visualized by their fluorescence. Since they are detected in a gel with no background fluorescence, the signal-to-noise level in the assay is improved compared to running samples in a buffer containing ethidium, it is commonly used for analysis of double stranded DNA fragments. Multiple sets of cleavage products from independent experiments can be analyzed on the same gel. Typically a single gel may be run 3-6 times for analysis of a total of 30 x 5= 150 samples. In contrast to agarose gels polyacrylamide gels can not be re-used for sequential analyses.

Since the agarose gels used in the present invention may be considerably shorter than standard agarose gels, they may be conveniently prepared in batches of about 5 gels by inserting multiple combs into a bed of unpolymerized agarose formed in a tray of conventional size (~20 X 16 cm). After the gel polymerizes, it is cut into multiple strips of ~4-5 cm with a razor blade. The labor, turnaround time, and cost of materials associated with gel preparation are substantially reduced, especially compared to the polyacrylamide gels used in conventional methods.

It is contemplated that nucleic acid staining agents other than ethidium bromide may be used in this assay. Such potential staining agents include, but are not limited to, Sybr Green I, Sybr Green II (Molecular Probes, Inc.), ethidium homodimer, and acridine orange.

Step 5. Analysis of results of the assay. In this step, the ethidium stained, electrophoretically separated cleavage products from Step 4 are viewed under ultraviolet light. The samples are scored as positive or negative for mutations based on the presence or absence of subfragments, detectable by eye or instrument, that are not present in the wildtype control lane.

In the common practice, where the most common phenotype in the test samples being analyzed is very likely to be the wildtype, it is not usually necessary to run a wildtype control. Since samples are generally scored as positive or negative based on the presence or absence of significantly smaller bands in test lanes, rather than on slight mobility differences between bands of equal size (as seen in SSCP), it is not necessary to intersperse wildtype control lanes at frequent intervals among the test samples.

The gels may be picked up and transferred to a uv-light box for viewing at any time (once the samples have migrated into the wells) and then replaced into the running buffer for further electrophoresis, for better resolution of larger fragments, if desired. Polyacrylamide gels, in contrast, cannot be easily monitored during electrophoresis.

It is contemplated that analysis of the nuclease cleavage products could be accomplished by automated means, instead of the direct visualization described here. The instrumentation for automated detection could include a photodetector, and/or a photomultiplier, and a recording device. Automated detection may offer significant advantages in terms of cost, speed of analysis, and especially, sensitivity. It is likely that current automated DNA sequencing instruments, such as those manufactured by Perkin-Elmer, Pharmacia, and Licor, could readily be adapted to this application.

D. Loading Buffers

In addition to providing a novel method for the analysis of the RNase cleavage products, the present invention also provides a novel use for certain reagents, which use significantly enhances the resolution of the cleavage products and increases the ability to detect mutations with the relatively low-resolution agarose gels. Using certain novel reagents in this manner also allowed the inventors to prepare a novel loading buffer composition that contains such reagents. Although this does not involve any newly synthesized chemical agents, the combination has a particularly advantageous property and such a combination has not been previously described.

The loading buffer composition of the present invention is a solution for use in separating double stranded nucleic acid fragments, especially RNA, on a non-denaturing gel, which solution has a high ionic strength. "High ionic strength" in this context means a loading solution that contains a concentration of salts, *e.g.*, sodium chloride or tetramethylammonium chloride (TMAC), sufficient to retard the mobility of the double-stranded RNA fragments and cause them to focus into sharp bands. It has been discovered that salt concentrations sufficient to provide a final salt concentration of at least about 0.5 M upon admixing with a sample are effective as high ionic strength loading solutions. Such solutions will also preferably contain the standard components, such as glycerol and bromophenol blue, as known to those of skill in the art and further described herein.

It is contemplated that further refinements may be made in the composition of the gel-loading buffer, for example the use of other salts or combinations of salts, inclusion of RNA-binding stains, alternatives to glycerol (for example sucrose or Ficoll or formamide) for imparting the required density to the sample, other tracking dyes instead of, or in addition to, bromophenol blue, *etc.* Such modified gel-loading solutions are held to be within the scope of this invention, as long as they contain concentrations of salts or other ionic compounds sufficient to cause improved resolution of double-stranded RNA or RNA/DNA duplexes in agarose gels.

E. Nucleic Acid Digestion Buffers

Another aspect of this invention is a nucleic acid digestion buffer comprising a pre-digested or pre-degraded protein mixture. As used herein the term "nucleic acid digestion buffer" refers to a mixture of components capable of cleaving a nucleic acid. Preferred embodiments of this invention encompass nucleic acid digestion buffers that allow specific RNase cleavage of base pair mismatches and are thus useful for detecting point mutations. The nucleic acid digestion buffer is preferably further defined as an RNA digestion buffer. An even more preferred embodiment encompasses a RNA digestion

buffer that is further defined as being adapted for cleavage of base pair mismatches in an RNA/DNA duplex or, most preferably, in double stranded RNA.

A preferred embodiment of this invention encompasses a nucleic acid digestion buffer, wherein the protein mixture is a digested protein mixture. A more preferred embodiment encompasses employing tryptone, peptone, casamino acids, N-Z amine, yeast extract, an acid or enzymatic hydrolysate of casein, an acid or enzymatic hydrolysate of soy bean, an acid or enzymatic hydrolysate of meat protein, or an acid or enzymatic hydrolysate of mixed protein as the digested protein mixture. Even more preferred embodiments use tryptone or peptone as the digested protein mixture. A further preferred embodiment is a nucleic acid digestion buffer, wherein the digested protein mixture is an acid or enzymatic hydrolysate of casein, soy bean, meat protein, or mixed protein. At present, most preferred embodiments employs a pancreatic digest of casein - tryptone.

Although the identity of the protein mixture will affect its optimal concentration, it is generally preferable to employ a nucleic acid digestion buffer, wherein the protein mixture is present in a concentration of between from about 0.2 to about 100 mg/ml. Concentrations of protein mixtures between about 1 and about 50 mg/ml are more preferred and between about 10 and about 40 mg/ml are the most preferred. The inventor presently employs 32 mg/ml or 16 mg/ml in stock buffers, which are diluted to 25.6 and 12.8 mg/ml respectively, during the preparation of the final reaction mixture for an assay.

A preferred embodiment encompasses a nucleic acid digestion buffer which further comprises an intercalating agent. The intercalating agent is preferably ethidium bromide, ethidium homodimer, acridine orange, or Sybr Green™, with acridine orange being more preferred and ethidium bromide being the most preferred. The intercalating agent is preferably present in concentrations as described previously for reaction mixtures of this invention.

Another preferred embodiment encompasses a nucleic acid digestion buffer, further comprising an RNase enzyme. Preferred RNase enzymes for nucleic acid digestion buffers are typically those employed in reaction mixtures of this invention as described above.

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F. Preferred Protein Mixtures

This invention teaches the use of a protein mixture in digestion buffers to enhance specific base pair mismatch cleavage. The protein mixture is preferably pre-digested and/or pre-degraded, such as an acid hydrolysate or enzymatic digest of casein protein, soy bean protein, meat protein, or mixed protein, or a crude extract containing such pre-digested protein components (for example, yeast extract). Enzymatic digests of such proteins may consist of digests with trypsin or pepsin. Such pre-digested proteins (for example, tryptone, peptone, casein hydrolysates, casamino acids, N-Z amine) are commonly sold as bacteriological media. These products may contain a mixture of amino acids, short peptides, ions, trace elements, and organic molecules, such as vitamins and co-factors.

A typical analysis of one such digested protein mixture, tryptone, which has been shown to be particularly useful in the present invention, is included herein in Table 1.

TABLE 1

ANALYSIS OF TRYPTONE - A PANCREATIC DIGEST OF CASEIN

ANALYTE	RESULTS
Physical Characteristics	
Ash (%)	6.8
Clarity, 1% Solution (NTU)	0.5
Filterability (g/cm^2)	1.3
Loss on Drying (%)	3.7
pH, 1% Solution	7.2
Carbohydrate (%)	
Total	7.7
Nitrogen Content (%)	
Total Nitrogen	13.0
Amino Nitrogen	5.2
AN/TN (%)	40.0
Amino Acids (%)	
Alanine	2.86
Arginine	3.03
Aspartic Acid	6.11
Cystine	0.42
Glutamic Acid	17.05
Glycine	1.75
Histidine	2.02
Isoleucine	4.40
Leucine	7.11
Lysine	6.70
Methionine	2.57
Phenylalanine	3.71
Proline	7.45

TABLE 1 (continued)

ANALYTE	RESULTS
Serine	4.29
Threonine	3.58
Tryptophan	0.71
Tyrosine	1.42
Valine	5.00
Inorganics (%)	
Calcium	0.013
Chloride	0.186
Cobalt	<0.001
Copper	<0.001
Iron	<0.001
Lead	<0.001
Magnesium	0.017
Manganese	<0.001
Phosphate	2.669
Potassium	0.229
Sodium	2.631
Sulfate	0.241
Sulfur	0.740
Tin	<0.001
Zinc	0.003
Vitamins (ug/g)	
Biotin	0.1
Choline (as Choline Chloride)	350.0
Cyanocobalamin	<0.1
Folic Acid	0.3
Inositol	1400.0
Nicotinic Acid	97.8

TABLE 1 (continued)

ANALYTE	RESULTS
PABA	3.7
Pantothenic Acid	5.3
Pyridoxine	0.6
Riboflavin	<0.1
Thiamine	0.4
Thymidine	93.4
Biological Testing (CFU/g)	
Coliform	negative
Salmonella	negative
Spore Count	73
Standard Plate Count	870
Thermophile Count	8

The list of components includes ions such as calcium, cobalt, iron, manganese, magnesium, copper, sulfur, tin, and zinc. Tryptone also typically contains vitamins, such as biotin, choline, folic acid, inositine, pyridoxine, thiamine, and riboflavin, and also co-factors such as nicotinic acid. Many of these molecules contain planar groups which may be capable of intercalating into double-stranded nucleic acid and altering its cleavage susceptibility.

Although preferred embodiments of this invention encompass employing tryptone as the protein mixture, this invention also incudes the use of a protein mixture other than tryptone. The inclusion of any of several different components normally used in the preparation of bacteriological growth media has been shown by the inventor to increase the ability of RNase I to effect double-strand cleavage of mismatches in duplex RNA substrates. For example, media other than tryptone which provide the beneficial effect include yeast extract (used at a concentration of about 5-15 mg/ml), casein hydrolysate, which is an acid hydrolysate of the milk protein casein (used at a concentration of about

10-30 mg/ml), N-Z Soy Peptone, Casein N-Z Amine, and Promotion, all products of Difco Laboratories, Detroit, Michigan, and all used at similar concentrations to tryptone, *i.e.*, 10-30 mg/ml.

The protein mixture may be added to the reaction mixture at an optimal concentration to increase the ability of RNases to effect specific cleavage of base pair mismatches, when admixed with the other reaction components. More particularly, the protein mixture may be present at similar or somewhat higher concentrations as those used for the growth of bacteria in culture, that is, from about 10 to about 40 mg/ml.

G. Preferred Divalent Cations

Another surprising aspect of this invention is the ability of divalent cations to enhance the specific cleavage of mutations if included in the digestion buffer. The divalent cations in the reaction mixture should be present at an optimal concentration to increase the ability of the RNase enzyme, for example RNase I, to effect specific cleavage of base pair mismatches, when admixed with the other reaction components. More particularly, calcium may be present at a concentration of from about 0.5 mM to about 10 mM. Other divalent cations, for example manganese, may be able to substitute partly or completely for calcium. This result is unexpected since RNase I does not require divalent cations for activity according to the scientific literature. (Zhu *et al.*, 1990).

H. Preferred Intercalating Agents

Another novel aspect of this invention is the increased ability to detect mutations *via* the addition of an intercalating agent to the digestion buffer. The intercalating agent is preferably ethidium bromide or a similar intercalating compound, for example, ethidium homodimer or acridine orange. The intercalating agent should be present in the reaction mixture at an optimal concentration to increase the ability of RNases to effect specific

cleavage of base pair mismatches, when admixed with the other reaction components. More particularly, ethidium bromide may be present at a concentration of from about 10 to about 200 μg/ml. This concentration is about 50-200-fold higher than the concentration (0.5 μg/ml) typically used to stain double-stranded nucleic acids for UV detection. Concentrations of ethidium providing between about 15 and about 150 mg/ml are more preferred, with concentrations of between 20 and 100 being most preferred. The inventor presently employs a stock solution with either 25 mg/ml or 100 mg/ml ethidium bromide, which is diluted to 20 mg/ml or 80 mg/ml respectively, during preparation of the final reaction mixture.

Embodiments of this invention also encompass using intercalating agents other than ethidium bromide. For example, specific cleavage of base pair mismatches in duplex RNA is improved by the inclusion of acridine orange, a common nucleic acid stain which intercalates between the bases in double-stranded RNA, in the RNase digestion mixture. Acridine orange was added at concentrations ranging from about 5 μg/ml to about 20 μg/ml, and shown to improve cleavage in 4 of the 5 mismatches tested. The fifth mismatch was cleaved even in the absence of an intercalating agent. See FIG. 7. One may also use intercalating agents such as ethidium homodimer, Sybr Green I™, and Sybr Green II™ (available from Molecular Probes, Inc. or through FMC Biotechnology Corp.) for this purpose. The preferred concentration of the various intercalating agents in the RNase digestion reactions may vary depending on the specific agent used, but will generally fall in the range of about 1 mg/ml - about 1000 mg/ml.

I. Amplification of Target Regions

The present invention may be useful for detecting mutations in nucleic acid sequences obtained by amplification of a target region by a variety of methods. These methods include amplification by PCR™ of genomic or mitochondrial DNA, amplification of mRNA or viral RNA by reverse transcriptase (RT)-PCR™, or amplification from

recombinant plasmids, phagemids, bacteriophage, or artificial yeast or human chromosome vectors and other methods known in the art.

The methods disclosed herein may have particular utility in the amplification of target regions from test and control samples from genomic DNA, cloned DNA, previously amplified DNA, or RNA. A particularly preferred method is the PCR™, with incorporation of exogenous promoter sequences into the amplified product by including phage promoter sequences in the oligonucleotide primers used in the amplification reaction. The promoter sequences are added in such a way that both strands of the amplified product can be transcribed into RNA *in vitro*. That is, promoter sequences are added to the 5′ ends of both the forward and the reserve PCR™ primers. The promoter sequences may be the same or different.

The target region may also be amplified from expressed sequences in test and control samples from mRNA isolated from a cell, mRNA transcribed *in vitro*, or from viral RNA. The test samples may be tumor tissue and the expressed sequences those from somatically mutated genes, or the test samples may be normal tissue with sequences expressed from genes containing germ-line mutations. The sequences may be expressed as mRNA and amplified for example by reverse transcription-PCR™, with incorporation of a label. The sequences may also be from viral RNA present in infected cells or tissue, or in extracts from infected cells or also tissue, or present in the virion particle itself. The test RNA sequences may also be unamplified endogenous mRNA or viral RNA.

In a kit form, a component for generating RNA may preferably comprise PCR product generated by use of a first and second PCR™ primer, each primer including a promoter sequences and a gene sequence from spatially separated regions of the same gene *e.g.*, the *p53* gene, and a RNA polymerase interactive with said promoter. The phage promoters, being small, of a single subunit, stable, and easy to produce are useful in the invention. The means for generating RNA may further comprises a second RNA polymerase interactive with said second promoter. An even more preferred embodiment

includes having the means of generating RNA further comprise a transcription buffer and a nucleotide solution. The means of generating RNA may further comprise a template gene sequence having the wild type sequence of said gene and a template gene sequence having the mutant sequence of said gene.

J. Production of Test Duplexes

The test duplexes may also be produced by hybridization of complementary strands of experimental and normal control nucleic acid, especially RNA, which are derived from recombinant plasmids or recombinant DNA molecules in vectors other than plasmids, such as phagemids, bacteriophage, or artificial yeast or human chromosome vectors. The complementary nucleic acid strands may be produced by *in vitro* transcription of the recombinant inserts using bacteriophage promoters contained in the vectors themselves, or by *in vitro* transcription of sequences amplified from the recombinant vectors using primers containing appropriate promoter sequences, for example bacteriophage promoters, such that the amplified products have promoters incorporated at their 5' ends. The recombinant inserts may be unmodified sequences cloned directly from sources such as genomic DNA, mitochondrial DNA, or expressed as mRNA or other cellular or viral RNAs (using reverse transcription to copy the RNA sequences into DNA to allow them to be cloned). Alternatively, the recombinant inserts may be the products of random or targeted mutagenesis schemes which introduce one or more mutations into the cloned sequences, either before or after they are inserted into the vector.

K. Use of Microtiter Plates

A preferred embodiment of the present invention includes detecting mutations in samples wherein the *in vitro* transcription reactions are carried out in microtiter plates. The transcription reaction components may be incubated for about 0.5-1 hour at 37 °C, and terminated by the addition of an equal volume of hybridization solution containing a

high concentration of formamide (for example, 80%). They may then be heated for about one minute at about 95 °C; the heating may be performed by placing the microtiter plate in a thermalcycler. Either prior or subsequent to heating the products of the *in vitro* transcription reaction, complementary experimental and normal transcripts may be mixed to make the duplex RNA substrates for RNase digestion.

The RNase digestion reactions may then be carried out in individual wells of microtiter plates, such as standard disposable U-bottom non-sterile polystyrene 96-well plates (sold by Dynatech Laboratories, Inc, 14340 Sullyfield Circle, Chantilly, Virginia, 22021; cat. no. 001-010-2205). Mixing of the duplex RNA substrates with the RNase solution may be accomplished by gently tapping and/or rotating the plates manually, or by specially designed mechanical vortexing devices. During incubation of the RNase digestion reactions, the wells of the microtiter plate may be covered with the adhesive film provided by the vendor with the plates, to prevent evaporation of the reactions. After incubation, the gel loading solution may be added and mixed with the RNase digestion reaction, and the contents of each well loaded on a native gel, typically a 2% agarose gel, and analyzed as in Example 8.

By performing the reactions in microtiter plates, the cost and labor associated with the assay is reduced, compared to carrying out the reactions in microfuge tubes. In addition, the feasibility of using automated devices, for example robotic arms, designed for high-throughput liquid handling, is increased. In contrast to the Non-Isotopic RNase Cleavage Assay described herein, the standard RNase cleavage-based mutation detection method requires organic extraction and alcohol precipitation of RNase cleavage products, and thus does not lend itself readily to being carried out in microtiter plates or to automation.

L. Detection Methods

Another preferred embodiment of the present invention encompasses the use of automated fluorescent photodetectors for analysis of RNase cleavage products, such as those used for automated DNA sequencing, to detect the size-fractionated RNase cleavage products generated. The photodetector and associated wavelength filters may be appropriate for detecting products which emit light of a given wavelength, for example in the orange-red range, when stained with ethidium bromide and excited with light in the ultraviolet range. If nucleic acid stains other than ethidium bromide are used, the specific detection properties of the photodetector, *i.e.*, the wavelengths at which the products are excited and detected, may be varied for their specific detection. The photodetector may be placed at a position, for example at the end of an agarose gel distal to that of sample loading, which is appropriate to detect electrophoretically separated subfragments of the duplex RNA substrates. Use of an automated fluorescent photodetector will facilitate the acquisition and processing of data required to fully automate genetic screening based on the Non-Isotopic RNase Cleavage Assay described herein.

The present invention may also be employed to detect mutations as cleavage products which are denatured and analyzed as single strands. The present methods enable the detection of mismatches by analysis of single stranded nucleic acid probes, especially RNA probes, which may be labeled by radioisotopes or by other means, or unlabeled, and hybridized to DNA or RNA targets according to the NIRCA™ method (U.S. Application Serial No. 08/371,531) or according to the method of Maniatis *et al.*, (U.S. Patent No. 4,946,773, 1990).

In a preferred method, after hybridization, the duplex nucleic acid targets may be treated with RNase(s) or other potentially appropriate nucleases, for example, S1 Nuclease or Mung bean nuclease, using the digestion conditions of the present disclosure as set forth in Example 8. After nuclease treatment, the nuclease may be inactivated, by protease digestion, by denaturation with SDS or guanidinium, or by other means. The

cleavage products may then be recovered, with alcohol precipitation, and the two strands denatured, by heating in a solution containing formamide. The cleavage products may be analyzed in a manner that maintains them as single strands by electrophoresis on a denaturing polyacrylamide gel. Cleavage products may be detected in a manner appropriate for the way in which they are labeled, by fluorescence under UV light or light of another wavelength, by autoradiography, by silver staining, or by secondary detection methods using binding and detection of streptavidin, as the case may be.

M. Reaction Mixtures For Improved Nucleic Acid Cleavage

A basic aspect of the present invention encompasses a reaction mixture comprising: an RNase enzyme; a nucleic acid intercalating agent; and an agent which enhances RNase mismatch cleavage activity, said agent comprising a digested protein mixture, a divalent cation, or both. As used herein the phrase "RNase mismatch cleavage activity enhancing agent" refers to a compound or mixture of compounds that increase the ability to detect mutations by increasing the specific cleavage of base pair mismatches.

1. The Digested Protein Mixture Component

A preferred embodiment of the invention is a reaction mixture wherein the RNase mismatch cleavage activity enhancing agent comprises a digested protein mixture. The digested protein mixture may comprise other components, for example, undigested protein.

An aspect of the present invention is improved mismatch cleavage due to including in the RNase digestion reaction mixture, crude tryptic or peptic digests of proteins, especially casein, or acid hydrolysates of proteins, especially casein, or yeast extract, or any of a wide variety of other crude amino acid-rich mixtures commonly used in the preparation of bacteriological media. For example, the digested protein mixture may be tryptone, peptone, casamino acids, N-Z amine, yeast extract, an acid or

enzymatic hydrolysate of casein, an acid or enzymatic hydrolysate of soy bean, an acid or enzymatic hydrolysate of meat protein, an acid or enzymatic hydrolysate of mixed protein, or brain-heart infusion with peptone and tryptone being more preferred. More preferred embodiments contain digested protein mixtures of casein, yeast, soy bean, meat protein, or other protein, with protein mixtures of an acid or enzymatic hydrolysate of casein being most preferred.

Most of the studies by the inventor used tryptone, a tryptic digest of casein, a protein found in milk although enzymatic hydrolysates of proteins derived from soy beans and meat are also effective. The particular combination of amino acids, short peptides, trace elements, ions, and organic molecules, such as vitamins and co-factors, found in such media, or a particular subset of such components, are highly effective for increasing the extent of specific mismatch cleavage by RNase I in a wide variety of double-stranded nucleic acid targets. The improved cleavage is most pronounced when the digestion mixture also contains ethidium bromide. The beneficial effect of the protein mixture for improving mismatch cleavage has not yet been seen when RNase T1 is used instead of RNase I, although the presence of this component is not detrimental to mismatch cleavage by RNase T1 and it is reasonable to expect that some effect may ultimately be seen.

The digested protein mixture may be present in a concentration of between about 0.2 and about 100 mg/ml. More preferably, the digested protein mixture is present in a concentration of between about 1 and about 50 mg/ml. In a most preferred embodiment, the digested protein mixture is present in a concentration of between about 10 and about 20 mg/ml.

Fractionation of the crude protein mixture (for example the casein hydrolysate, tryptone, N-Z Amine, yeast extract, *etc.*) may permit critical components increasing nucleic acid cleaving activity to be identified, allowing the benefits of the present invention to be duplicated using a more well-defined system. However, reconstitution of

a defined system may be more difficult, more costly, and no more effective than the present crude mixtures for achieving maximum mismatch cleavage efficiency by RNase I. A detailed analysis of the various components comprising a typical lot of tryptone is included herein to illustrate the factors, including but not limited to ions, vitamins, and co-factors, that are in fact present in this type of digested protein mixture (See Table 1).

2. The Divalent Cation Component

A further preferred embodiment of this invention employs a divalent cation as the RNase mismatch cleavage activity enhancing agent although the scientific literature states that divalent cations are not required for RNase I cleavage of single-stranded RNA substrates (Zhu *et al.*, 1990). Indeed, RNase I activity on single-stranded substrates is typically assessed in the presence of the divalent cation-chelating agent, EDTA (Meador and Kennell, 1990; Gesteland, 1966), so that free divalent cations are not present in the RNase I reaction mixture. Quite surprisingly, the present inventor has found that RNase I cleavage of some mismatches is dramatically improved by including certain divalent cations, for example, calcium, manganese, or a combination of calcium and manganese. FIG. 6, FIG. 8A, FIG. 8B, FIG. 8C, FIG. 8D, and FIG. 8E show the effects of Ca^{++}. It is likely that other divalent cations will also show this effect.

A more preferred embodiment employs Ca^{++} as the divalent cation. In this embodiment Ca^{++} is preferably present in a concentration of from about 0.1 mM to about 100 mM, with concentrations of from about 0.5 to about 25 mM being more preferred and concentrations of from about 1.5 to about 5 mM being most preferred.

The optimum concentration(s) of divalent cation(s) to use in the RNase digestion mixture may depend on the specific concentrations of the other components, for example, sodium chloride or other monovalent cation(s), ethidium bromide, EDTA, and RNase I. Without being bound by any particular theory, the inventor hypothesizes that the effect of

the ions in the reaction is probably due partly to an effect on the RNase and partly to an effect on the target duplex.

3. Useful Intercalating Agents

The invention often takes the form of a reaction mixture having an intercalating agent, for example, ethidium bromide, ethidium homodimer, acridine orange, or Sybr Green™ Of the intercalating agents acridine orange is preferred and ethidium bromide is even more preferred. In preferred embodiments, the intercalating agent is present in a concentration of from about 1 to about 1000 μg/ml. It is more preferred to employ a concentration of intercalating agent of from about 10 to about 100 μg/ml.

Intercalating agents are planar molecules which bind to double-stranded nucleic acids between the nucleotide bases in single and double-stranded RNA and DNA. Ethidium bromide is known to those of skill in the art as an intercalating agent that is a sensitive stain for detecting nucleic acids. However, ethidium bromide has not previously been used as a component of an RNase digestion reaction specifically added to improve mismatch cleavage. Another intercalating agent used to stain nucleic acids, acridine orange, can be also used to improve RNase I cleavage of some base pair mismatches in duplex RNA (FIG. 7).

The finding by the present inventor that intercalating agents, such as ethidium bromide, increase the ability of RNase I to cleave base pair mismatches under certain conditions was unexpected, and not seen with RNase A to the same frequency as is seen for RNase I. The specific mismatch cleavage capability of RNase T1 is also dramatically improved by high concentrations of ethidium bromide (FIG. 5). Unlike the situation with RNase A and RNase I, inclusion of ethidium bromide in the RNase T1 reaction does not require a concomitant reduction in the enzyme level to prevent over-digestion.

Carefully optimized levels of ethidium bromide have so far proven most effective for improving mismatch cleavage. Merely adding ethidium bromide to standard RNase mismatch cleavage reactions using RNase I or RNase A may result in nearly complete non-specific degradation of the target duplex. High levels (higher by about 50-200 fold) of ethidium bromide are needed in one of the preferred embodiments of the present invention (another preferred embodiment uses 25 μg/ml instead of 100 μg/ml), compared to the ethidium bromide concentrations used in the routine detection of nucleic acid (100 μg/ml in the present invention versus 0.5 μg/ml for routine UV detection). The ability to use ethidium bromide at the high levels required for optimal mismatch cleavage, without non-specifically degrading the target duplex, is achieved by a concomitant reduction in the concentration of the RNase (RNase A or RNase I) used for mismatch cleavage.

The mechanism by which intercalating agents, such as ethidium bromide, improves RNase mismatch cleavage is unknown, but probably the effects of ethidium bromide on the mismatched substrate itself are more important than effects on the nucleases. While not wishing to be bound by any particular theory, the intercalation of ethidium bromide into duplex nucleic acid presumably alters its conformation such that mismatches are more susceptible to cleavage. The increased susceptibility may be due to more pronounced helical distortion at the position of the mismatch, to a general loosening of the hydrogen bonds holding the two strands of the duplex together, or even to the recently described phenomenon of "base flipping" (Roberts, 1995). Additionally, ethidium bromide also improves RNase degradation of single-stranded RNA by approximately three times. That is, about one third the amount of the RNase that is required to degrade the single strand substrate in presence of ethidium is required when ethidium bromide is present.

4. Enzymes

The digestion buffers of the present invention allow for a broader choice of enzymes to cleave mismatches. For example, under the standard reaction conditions used in the prior art for performing the RNase digestion step in RNase mismatch cleavage assays, RNase A has heretofore been the only ribonuclease generally thought to be useful for mismatch cleavage.

A preferred embodiment of this invention is a reaction mixture, wherein the RNase enzyme is RNase I, RNase I* from yeast, RNase A, modified RNase A, RNase B, a mixture of RNase A and RNase B, RNase T1, in some embodiments RNase I is presently most preferred.

The RNase enzyme is typically presently employed in a concentration of from about 0.01 μg/ml to about 500 μg/ml. A concentration of from about 0.1 μg/ml to about 250 μg/ml is even more preferred. Of course, different RNases work best at different concentrations. Most preferred embodiments relevant to specific RNases include a reaction mixtures, wherein the RNase enzyme is RNase A in a concentration of from about 0.01 μg/ml to about 0.5 μg/ml, with the inventors presently employing 0.3 μg/ml; RNase I in a concentration of from about 0.1 μg/ml to about 0.3 μg/ml; or RNase T1 in a concentration of from about 200 μg/ml to about 300 μg/ml, with the inventors presently employing 250 μg/ml.

The advantages of the invention become apparent when looking at the effects of the media on (see Fig. 6) particular RNases.

The present invention allows RNase I to be used for the detection of mismatches. The addition of calcium ions to the RNase I digestion mixture can substitute to a large extent for the protein mixture component, as shown in FIG. 8A, FIG. 8B, FIG. 8C, FIG. 8D, and FIG. 8E, and the beneficial effect of the protein mixture can be eliminated by

addition of EDTA or EGTA, agents which chelate divalent cations (EDTA) or which preferentially chelate calcium ions (EGTA). Therefore, it is postulated that calcium ion may be one critical component of the protein mixture that confers improved mismatch cleavage by RNase I in some embodiments of the invention. A beneficial effect of divalent cations such as calcium and manganese for mismatch cleavage using RNase A or RNase T1 has not been detected. The inventor has not yet duplicated all of the beneficial effects of a protein mixture component on the cleavage of the broadest range of different mismatches by the mere alternative use of calcium in the RNase digestion mixture. That is, although most mismatches tested which are cleaved by RNase I are cleaved equally well in the presence of optimized levels of calcium and ethidium bromide, compared to optimized levels of tryptone and ethidium bromide, there are some examples of mismatches which are cleaved better in the presence of the tryptone/ethidium bromide mixture.

One particular advantage of the present invention is that it allows for the use of RNase I at much lower concentrations than the methods described in the literature. The amount of RNase I required for optimal cleavage in the present composition is approximately 300-60-fold less than that used in previously described methods. This results in a substantial reduction in the cost of consumable materials needed to carry out the assay. In fact, using the present invention, RNase I appears to surpass RNase A in its ability to cleave base pair mismatches in double-stranded RNA. In contrast, prior studies by the inventor not employing the present invention found that RNase I was inferior to RNase A for mismatch cleavage in both RNA/RNA and RNA/DNA targets. Under the standard RNase digestion reaction conditions used in the prior art, many more of the mismatches in the Factor IX mutants tested in a model system were cleaved by RNase A than were cleaved by RNase I.

RNase T1, isolated from *Aspergillis orysae*, which has not generally been considered a useful enzyme for mismatch cleavage (Myers *et al.*, 1985), is able to specifically cleave many mismatches when used under the conditions of the present

invention (FIG. 5). The inventors are aware of only a single instance in the literature that describes RNase T1 being used for cleavage of a basepair mismatch. In that experiment, analysis of the cleavage products was according to the original method (Marquadt *et al.*, 1991 Maniatis *et al.*, U.S. Patent 4,946,775, 1990) and not according to the NIRCA™ method presently preferred by the inventors (as described in U.S. Patent application SN 08/371,531). Also, that experiment did not employ several of the inventive aspects of the present invention, for instance, no intercalating agent or mismatch cleavage activity enhancing agent such as digested protein or calcium, was used. RNase T1, which is widely known to be specific for cleavage of guanosine residues in single-stranded RNA (see Worthington Enzymes and Biochemicals Manual, 1993, and references therein), had previously been tested for its ability to cleave mismatches containing guanosines in RNA/RNA and RNA/DNA duplexes. No mismatch cleavage was detected previously using this enzyme. That result was in agreement with that of Myers *et al.* (1985), who also reported that RNase T1 was not able to generate detectable mismatch cleavage products in RNA/DNA targets under various conditions.

Other RNases, namely the cytoplasmic RNase isolated from yeast (Cannistraro and Kennell, 1991) and the bacterial cytoplasmic RNase known as RNase I*, isolated from the cytoplasm of *E.coli*, (Cannistraro and Kennell, 1991) have also been tested and found to be capable of cleaving single base pair mismatches in duplex RNA, and potentially also in RNA/DNA duplexes, especially when used in conjunction with the other novel reaction components of the present invention. It is likely that other bacterial RNases, for example, the periplasmic RNase isolated from *Aeromonas hydrophila* (Favre *et al.*, 1993), will also be useful for mismatch cleavage in the reaction mixtures of the present invention.

Although not all known eukaryotic and prokaryotic ribonucleases have been tested for improved ability to cleave mismatches when used according to the present invention, the advantages of the present invention are expected to be general, because the activity

of all RNases which have been tested (both eukaryotic and prokaryotic) have been found to be improved.

One of the most important aspects of the RNase digestion conditions disclosed in the present invention is that they result in a significantly higher mutation detection rate than has been previously reported using the RNase mismatch cleavage assay. Detection is improved by using RNases which do not reliably cleave base pair mismatches under standard conditions. RNases found to be especially improved by the present invention in their ability to cleave mismatches include RNase I and RNase T1. However, the invention reduces the amount of non-specific cleavage seen with RNase A, and reduces the amount of RNase A needed for specific cleavage, when RNase A is used in the mismatch cleavage assay.

Of the three mutations in the Factor IX model system that were not detected by either RNase A alone or the RNase A/RNase I combination used in the initial studies, all are detected using the new RNase digestion conditions disclosed in the present invention. Mutations in the *p53* tumor suppressor gene that are not detected by RNase A are also detected by RNase I using the new conditions (FIG. 9A, FIG. 9B, and FIG. 9C). Moreover, when the entire panel of 60 mismatches in the model system (2 complementary mismatches are generated from each of the 30 point mutations) is compared using the new RNase digestion components and the components used in the method described in U.S. Patent by Maniatis et al 4,946,775, it is clear that the new conditions show a dramatic improvement in the general ability to specifically cleave a wide variety of mismatches.

There are some examples of mismatches which are cleaved better by RNase A than RNase I, when the conditions of this invention are used. Part of the general improvement seen in the RNase mismatch cleavage assay using the conditions disclosed herein is due to the reduction in non-specific cleavage seen when using RNase I instead of RNase A. Even when RNase A is used instead of RNase I, the background of non-

specific cleavage is reduced by using the RNase digestion reaction components specified in the present invention. Therefore, a general property of the invention seems to be the ability to reduce the effects of non-specific background cleavage. This results in a lower background and improved signal-to-noise ratio in the assay. The general reduction in non-specific cleavage can make it feasible to mix together two or more RNases (*e.g.*, RNase A and RNase I) to use in mismatch cleavage assays. This would reduce the labor involved (compared to using the RNases in separate readiness) and potentially improve cleavage of some mismatches.

5. Other Components

Another embodiment of the invention encompasses a reaction mixture that further comprises monovalent cations, such as NaCl. When employed the sodium chloride is preferably present in a concentration of from about 5 to about 300 mM. A concentration of from about 50 to about 200 mM is more preferred, with a concentration of from about 15 to about 100 mM being most preferred.

The RNase digestion reaction mixture may also contain additional ingredients, for example magnesium chloride, Tris-buffer, EDTA, *etc.* These components may be useful for achieving optimal sensitivity and specificity of mismatch cleavage. For example, if the NaCl concentration falls below a threshold level, non-specific cleavage increases dramatically, especially when RNase A or RNase I is used. This effect can be counteracted by reducing the ribonuclease concentration. Adding ethidium bromide to a reaction previously balanced for NaCl and RNase concentration can also increase non-specific cleavage dramatically, especially when the RNases used are RNase I and/or RNase A.

The optimal concentration of the digested protein component tends to be more constant between reactions that differ widely in the levels of NaCl, RNase, and ethidium bromide. Because optimal levels of the reaction components vary depending on the

concentrations of other components, two different examples of compositions containing the digested protein mixture are provided of some of the preferred embodiments of the invention for illustrative purposes only. In one composition, the monovalent cation (NaCl) has been eliminated, although residual sodium (about 20 mM) is provided by the predigested protein (*i.e.*, tryptone) component.

6. Nucleic Acids

The reaction mixture of the present invention typically further comprises a nucleic acid. The nucleic acid may be a double stranded nucleic acid. The double stranded nucleic acid may be a double stranded RNA, an RNA/DNA duplex or a double stranded DNA. Most NIRCA™ based aspects of the invention employ a double stranded RNA.

7. Examples of Reaction Mixtures

One general composition for a reaction mixture effective for cleaving base pair mismatches in duplex nucleic acid targets would contain an RNase with specificity for single-stranded regions of duplex RNA targets; an intercalating agent present at 0.5-50 μg/ml; and a mismatch cleavage activity enhancing agent such as a digested protein mixture and/or calcium ion. The digested protein mixture may contain trade contaminants which are effective for providing the mismatch cleavage activity enhancing agent. The composition may also contain additional ingredients such as sodium chloride or other monovalent cations, formamide, Tris buffer or other physiological buffer, detergents such as SDS, magnesium ion, or other components carried over from the *in vitro* transcription reaction used to produce the transcripts that are hybridized to make the duplex RNA target.

A specific example of a reaction mixture of the present invention may contain the following components: tryptone (preferably about 25 grams/liter); ethidium bromide (preferably about 25 micrograms/ml); sodium chloride (preferably about 85 millimolar), and

RNase I (preferably about 0.5-1 unit/microliter), or RNase A, (preferably at a concentration of about 0.5 nanograms/microliter), or RNase T1 (preferably at about 64-160 units per microliter). Instead of tryptone, the reaction mixture may contain calcium chloride (preferably about 1.5 - 10 millimolar).

Alternatively, the reaction mixture may contain: tryptone (preferably about 25 grams/liter); ethidium bromide (preferably about 80 micrograms/ml); and RNase I (preferably from about 0.1 unit to about 0.33 units per microliter), or RNase A, preferably at about 0.1 nanograms per microliter, or RNase T1 (preferably at about 64-160 units per microliter). A "digestion reaction" is usually prepared by a user from a stock nucleic acid digestion buffer solution which is diluted during preparation of the reaction mixture. The inventor currently envisions that a commercial stock buffer solution might comprise 100 μg/ml ethidium bromide and 32 mg/ml tryptone, which will be diluted to final concentrations of 80 mg/ml and 25.6 mg/ml, respectively, during the preparation of the digestion reaction.

For cleavage of base pair mismatches by RNase T1, the above reaction mixtures may be used, or the tryptone or digested protein or calcium ion component may be omitted from the reaction mixture. The requirement for the digested protein or calcium ion component for optimal cleavage of mismatches with RNase I does not appear to apply to RNase T1, at least not with those mismatches tested thus far. It is possible that examples will be found in the future of mismatches which are cleaved better by RNase T1 when the digested protein and/or calcium ion components are included in the reaction mixture. The RNase T1 may be present at a concentration of about 64-160 units per microliter, corresponding to a mass concentration of about 100-250 micrograms per milliliter. The preferred concentration of ethidium for mismatch cleavage by RNase T1 is from 25-100 micrograms per milliliter, especially 100 μg/ml.

In a preferred embodiment, one volume of solution containing the double-stranded nucleic acid substrate may be mixed with four volumes of one of the above mixtures and

incubated for 30-60 minutes at 37 C. After the incubation, gel-loading buffer may be added and the RNA cleavage products may be analyzed by the NIRCA™ techniques described.

The concentrations of each of the above reaction components may generally be varied over at least a several-fold range and still provide an effective composition for cleavage of base pair mismatches in double-stranded RNA and presumably also in RNA/DNA targets. Although cleavage of base pair mismatches in DNA/DNA targets would not be expected to occur using RNases, it is possible that this invention may also improve cleavage of such mismatches by DNases or non-specific nucleases such as S1 or mung bean nuclease.

In some cases, the concentrations of the above components may be varied independently over appropriate ranges. For example, the digested protein and calcium components may be varied over a several-fold range from that stated in the preferred embodiment, and still provide an effective concentration for improving mismatch cleavage by RNases.

For other components, varying the concentration of certain components may require that the concentration(s) of other components be varied concurrently to maintain the optimal composition for specific mismatch cleavage. For example, the RNase I concentration may be increased to 5 units/microliter, and the ethidium bromide concentration concurrently decreased to about 10 micrograms/milliliter, while still providing a composition effective for the specific cleavage of base pair mismatches in duplex RNA targets. If the RNase I concentration is increased beyond about 5 units/microliter, the ethidium bromide concentration must be further decreased in order to prevent non-specific digestion of the target duplex. If the sodium chloride and/or EDTA concentrations are increased or decreased, the concentrations of ethidium bromide and/or tryptone and/or calcium and/or RNase enzyme (especially RNase I and/or RNase A) may

be adjusted to compensate for the resulting altered stability of the target duplex, and the resulting altered concentrations of monovalent cation in the reaction mixture.

The optimal levels of the various reaction components may also be dependent on the nucleotide composition and sequence of the specific target region being analyzed. For example, higher RNase I levels and/or lower sodium chloride levels may be required for cleaving mismatches in G+C-rich targets, compared to A+U-rich targets.

N. Methods of Cleaving and Detecting Base Pair Mismatches

Another aspect of this invention encompasses a method for detecting a base pair mismatch in a nucleic acid molecule; comprising the steps of: obtaining a single stranded nucleic acid test sample to be analyzed, preferably RNA; contacting said RNA test sample with a single stranded nucleic acid probe thereby forming a test duplex; treating said test duplex with a ribonuclease composition capable of cleaving double-stranded nucleic acid molecules containing base pair mismatches under conditions effective to allow the formation of cleavage products, said ribonuclease composition comprising: an RNase enzyme; and, in some preferred embodiments a nucleic acid intercalating agent; and/or an RNase mismatch cleavage activity enhancing agent comprising a protein mixture - preferably a digested protein mixture, a divalent cation, or both; and separating said cleavage products under conditions that allow the cleavage products to remain double-stranded.

1. The Test Nucleic Acid Duplex

The "test nucleic acid duplex" is a double stranded nucleic acid that is to be assayed for the presence of base pair mismatches. For example, this is the sample to be screened for mutations if this method were employed to screen patients for various diseases or conditions characterized by nucleic acid mutations. The double stranded

nucleic acids, RNase mismatch cleavage activity enhancing agents, RNase enzymes, and intercalating agents employed in this aspect of the invention are as described previously.

2. RNA Test Sample

In this aspect of the invention the RNA test sample is reacted with a nucleic acid probe, typically of known nucleic acid sequence and preferably a single stranded RNA, such that a nucleic acid duplex is formed by complementary interactions between base pairs. The ribonuclease composition of this aspect should be designed to specifically cleave base pair mismatches. Therefore, reacting the nucleic acid duplex with the ribonuclease that cleaves at mismatches allows for the determining of mutations due to mismatches between the test sample and the nucleic acid probe. The elements of the preferred ribonuclease composition are as described previously for reaction mixtures and nucleic acid digestion buffers of this invention.

In preferred embodiments of this aspect, the RNA test sample and RNA probe are prepared by transcription of a recombinant plasmid or a PCR™ product. It is even preferable to prepare the RNA test sample and RNA probe simultaneously in the same reaction by *in vitro* transcription.

The RNA test sample, the RNA control sample, or both, may be prepared by transcription of a recombinant plasmid or by transcription of a PCR™ product. In a preferred embodiment, the template for the *in vitro* transcription reaction will be one that contains opposable promoters, *i.e.*, promoters that interact with the same or different RNA polymerase enzymes in order to effect transcription of both strands of the template DNA into RNA. Examples of preferred promoters are the SP6, T7 and T3 promoters, any combination of which may be employed. In a form of the method that involves an internal control, the opposable promoters used may be the same, allowing transcription by a single RNA polymerase.

In providing a control, the method of detecting a base pair mismatch in a double-stranded RNA molecule may generally comprise the steps of: (a) obtaining a single stranded RNA test sample to be analyzed and a single stranded RNA non-mutant control sample; (b) contacting the test RNA sample and the non-mutant control RNA sample with a single stranded RNA probe, thereby forming a test RNA duplex and a non-mutant control RNA duplex that is free from mismatches; (c) treating the test RNA duplex and the non-mutant control RNA duplex with a ribonuclease composition capable of cleaving double-stranded RNA molecules containing base pair mismatches, under conditions effective to allow the formation of double stranded cleavage products; (d) separating the cleavage products under conditions that allow the cleavage products to remain double-stranded; and (e) comparing the separated double stranded cleavage products from the test RNA duplex and the control RNA duplex, wherein a difference in the size of the cleavage products is indicative of the presence of a base pair mismatch in the original RNA test sample.

The RNA test sample may be purified from a cellular extract or obtained from a biological sample from a patient suspected of having a disease associated with a genetic mutation. To screen for genetic diseases, an RNA transcript from a normal gene ("probe") is hybridized to an RNA transcript from the test gene (*i.e.*, from a patient sample), to form a duplex RNA target molecule. Regions of non-complementarily between the probe and the test transcripts will result in base pair mismatches of one or more bases in the duplex RNA target molecule. The target molecule is then reacted with a mixture comprising an RNase capable of cleaving unpaired residues ("mismatched") in the target molecule, and agents which enhance the ability of the RNase to effect said cleavage. The cleavage products are separated according to size by electrophoresis or other means and analyzed by comparison to cleavage products from a control duplex RNA molecule ("no-mismatch control") produced by hybridizing of complementary transcripts from the normal gene and similarly treated with RNase. Differences in the sizes of the cleavage products are indictable of mutations in the target molecule. The size differences are due to cleavage by RNase at the regions of non-complementarily between the normal

transcript and the transcript from the test gene. The regions of non-complementarily are due to mutations in the test gene.

To allow generation of a large mass of RNA, it is currently preferred that the RNA test sample be prepared by transcription of a PCR™ product. Indeed, the RNA test sample, RNA control sample and RNA probe are all preparable by transcription of a PCR™ product. The RNA test sample and control sample may be prepared simultaneously in the same reaction by *in vitro* transcription.

Irrespective of the manner in which the RNA target is obtained or prepared, the original source of the RNA may be a sample, including a blood or biopsy sample, obtained from a patient suspected of having cancer, hemophilia, or any other such mutation-associated diseases, as are known in the art.

Although not required by any means, one component of the test or control RNA duplexes in the assay may include a non-radioactive label. Exemplary non-radioactive labels include fluorescein and rhodamine. Even a radioactive label could be employed if desired, although it is a distinct advantage of the invention that this is not required.

The test or control samples may also be conjugated or otherwise associated with, *i.e.*, captured on a solid support prior to treatment with the ribonuclease composition.

3. Separation of Cleavage Products

A preferred embodiment of this aspect includes separating the cleavage products using non-denaturing gel electrophoresis. The cleavage products may preferably be admixed with a high ionic strength loading solution to form a loading sample prior to separation by non-denaturing gel electrophoresis. In even more preferred embodiments the loading solution comprises a salt in a concentration sufficient to provide a final salt concentration in each loading sample of at least about 0.5 M. The loading solution

preferably comprises a tetramethyl alkyl salt, such as tetramethylammonium chloride, or NaCl.

Alternatively, it may be preferable to separate the cleavage products using non-denaturing HPLC or capillary electrophoresis.

4. Detection of Cleavage Products

The cleavage products may be detected by methods known of skill in the art. For example, they may be contacted with an agent that causes the cleavage products to fluoresce, such as ethidium bromide. This agent may be incorporated into the nondenaturing gel or added to the sample prior to electrophoresis. The cleavage products may also be analyzed by silver staining or using an automated device.

D. A Kit for Use in Detecting Base Pair Mismatches and/or Base Changes in a Nucleic Acid Sequence

An embodiment of this invention is a kit for use in detecting base pair mismatches, the kit comprising, in a suitably aliquoted form, a component for generating RNA and an RNase enzyme. The kit may also include an RNA digestion buffer comprising a digested protein mixture, a divalent cation, or both and an intercalating agent. The preferred RNA digestion buffers are as previously described for reaction mixtures and nucleic acid digestion buffers.

The enzyme or enzymes may be provided in lyophilized, powdered form to be stored in a freezer at about -20°C to about -80°C and to be mixed with solvents in the indicated concentrations just prior to use, or alternatively, the enzyme or enzymes may be provided in solution in an appropriate buffer and possibly glycerol solution. All of the said solvents and buffers may or may not be provided with the kit.

The kits will generally also contain a high ionic strength gel-loading solution for use in significantly enhancing the resolution of the RNase cleavage products. Such a solution will contain, in addition to the standard components, a concentration of a salt sufficient to retard and to focus double-stranded nucleic acid fragments into sharp bands. Suitable salts are sodium chloride and tetramethylammonium chloride (TMAC). An example of a high ionic strength gel-loading solution is 3 M TMAC, 10 mM Tris 7.5, 2 mM EDTA, 0.1% bromophenol blue, 50% glycerol. The buffer may be provided as a premixed solution or as various aliquots of components for forming such a solution.

Other components of the kit may include, but are not limited to, components for preparing a non-denaturing gel, a solution of enzyme buffer and/or a solution of NaCl. The kit may include a digestion buffer comprising a digested protein mixture, a divalent cation, and/or an intercalating agent. Sterile, nuclease free, purified water may also be included in the kit. The kit may also contain a control RNA template(s) with and/or without a mismatch with a complementary RNA probe. The RNA molecules will be provided in solution or in lyophilized form and would be stored at 4°C or alternatively could be stored at -20°C to -80°C.

The kit may also contain PCR™ primers to be used to amplify the test sample. The primers will preferably be comprised of phage promoter sequences to be used in the transcription of the PCR™ products. The primers may also comprise sequences complementary to the regions of test sample being amplified. The phage promoter sequences are appended to the 5' ends of the test gene-specific sequences.

A specific example of a kit in accordance with the invention is that which includes a 10X transcription buffer and rNTP mix; SP6 and T7 RNA polymerases (20 Units/ml each); a hybridization solution; an RNase stock solution and digestion buffer; the afore-mentioned improved gel loading solution; RNase-free dH_2O; a wild type and mutant control template, *e.g.*, for the *p53* gene; and PCR™ primers (amplimers) with phage promoters.

P. Methods of Screening For an RNase Mismatch Cleavage Activity Enhancing Agent

Another embodiment of the present invention is a method of screening for an RNase mismatch cleavage activity enhancing agent comprising: obtaining a digested protein mixture; fractionating the digested protein mixture into two or more fractions; and testing a fraction of the digested protein mixture for RNase mismatch cleavage activity enhancing characteristics. Preferred digested protein mixtures are as described previously for other embodiments of this invention.

A preferred embodiment of this invention is the method of screening for an RNase mismatch cleavage activity enhancing agent, wherein the step of testing the fraction comprises: preparing a test RNA digestion buffer comprising the fraction and an RNase enzyme; preparing a control RNA digestion buffer substantially identical to the test RNA digestion buffer and comprising the same RNase enzyme, but not having the fraction; performing parallel tests with both the test RNA digestion buffer and the control RNA digestion buffer to determine the relative activities of the RNase enzyme in the test and control buffers. Preferred RNase enzymes in the practice of this aspect of the invention are as described previously.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

II. EXAMPLES

EXAMPLE 1

Non-isotopic RNase Cleavage Assay

In performing an RNase cleavage assay in accordance with this invention, the inventors currently prefer to use certain compositions and steps, which are set forth in the present example.

Amplification of target regions from test and control samples. The samples may be genomic DNA, cloned DNA, previously amplified DNA, or RNA. The samples may be obtained by, for example, PCR™, with incorporation of exogenous promoter sequences into the amplified product, for example, by including phage promoter sequences in the oligonucleotide primers used in the amplification reaction. The promoter sequences are added in such a way that both strands of the amplified product can be transcribed into RNA *in vitro*.

Transcription of both strands (sense and antisense) of the test and control target regions into RNA. In a preferred embodiment, transcription reactions contain mass amounts of unlabeled ribonucleotides sufficient to generate several micrograms of transcript at a concentration of about 100-500 μg/ml. For example, the *in vitro* transcription reactions could contain:

A. 2 μl PCR™ template with opposable promoters, for example, T7 and SP6 promoters
B. 1μl of buffer containing salts, spermidine, and buffering and reducing agents
C. 2 μl of mixture of 2.5 mM all 4 ribonucleotide triphosphates
D. 4 μl dH_2O
E. 1 μl of 20 U/μl RNA polymerase, eg. T7 or SP6 RNA Polymerase

The template for the *in vitro* transcription reaction may comprise a mixture of amplified products from test and wildtype control target regions. The test and wildtype control target regions may both exist in a single sample amplified from a heterozygous source. The RNA polymerase used may be a mixture of polymerases, for example T7 and SP6.

The reactions may be incubated and terminated according to standard procedures. In a preferred embodiment, they are terminated by heating in an equal volume of buffer containing a high formamide concentration and low salt concentration, for example 80% formamide/25 mM Sodium citrate pH 6.8.

Mixing Sense and Antisense products of the *in vitro* transcription reactions such that complementary transcripts derived from wildtype control and test target regions are able to hybridize and form double-stranded nucleic acid molecules, in particular RNA-RNA molecules. The complementary Sense and Antisense transcripts derived from the wildtype control template are also mixed and hybridized. In a preferred embodiment, at least 2 μl of each transcription reaction is mixed with 2 μl of its complement, heated briefly (~3 minutes) at 80-100°C, and allowed to cool to ambient temperature over a period of several minutes. In the case where the template for the transcription reaction included a mixture of control and test target templates, this step may not be required.

Treating the hybridized wildtype control and test transcripts (RNA test duplexes), and also the hybridized complementary wildtype control transcripts (RNA control duplexes), with nuclease(s) or other agents, for example chemicals, such that cleavage of RNA target duplexes containing base pair mismatches takes place to generate products which are altered in size compared to the RNA control duplex treated in the same way. In a preferred embodiment, the volume of solution (for example nuclease solution) added to the RNA duplexes should be sufficiently small that the final reaction products can be analyzed directly on a gel. For example, 16 μl of a solution containing a nuclease

capable of cleaving base pair mismatches could be added to 4 μl of an RNA duplex, mixed, and incubated at 37°C for 30 minutes.

Analyzing the treated RNA duplexes to detect cleavage products of altered size in the test compared to the control duplex, in such a way that the products remain double-stranded during the analysis. In a preferred embodiment, a small amount (~ 1/6-1/10 volume) of a dense loading solution is added to all samples, which are then loaded directly, without heating, onto a gel. The nucleic acid fragments in each sample are separated by electrophoresis according to size. For example, the gel could be run at 80 volts for 30 minutes in a buffer containing 1X TBE. The gel could include 2% agarose in 1X TBE containing 0.5 μg/ml ethidium bromide.

The fragments are visualized by staining with a dye that binds to double-stranded nucleic acid, for example ethidium bromide. Staining may take place before and/or during separation of the fragments, for example, by adding ethidium bromide to the sample and/or the gel and/or the buffer, or staining may take place subsequent to the separation, for example by soaking the gel in solution containing ethidium bromide. The dense loading solution may contain ingredients that cause the nucleic acid fragments to migrate as sharp bands, rather than diffuse bands, which would be difficult to analyze. For example the dense loading solution can contain tetramethylammonium chloride and ethidium bromide. After electrophoresis, the gels are examined under ultraviolet light.

Test samples containing nucleic acid fragments which are not seen in the control reaction are scored as positive for containing base pair mismatches, which result from mutations in the test target region being screened.

EXAMPLE 2

Comparing the Conventional RNase Mismatch Cleavage Assay with the Present Non-isotopic RNase Mismatch Cleavage Assay

Materials and Methods

Conventional RNase Protection Assay

^{32}P-labeled 401 nt Sense and Antisense transcripts (specific activity $\sim 10^6$ cpm/μg) were transcribed from a PCR™ template containing opposable phage promoters. The second-step PCR™ primers include T7 and SP6 phage promoters at their 5′ and 3′ ends, and were added by overlap extension to the products of the first PCR™, which included a fragment amplified from the 5′ half of exon 8 of the Factor IX gene. The Sense and Antisense transcripts were mixed separately with about 100 ng of PCR™ products amplified from blinded samples of genomic DNA from normal controls and Hemophilia-B patients.

The RNA probes and DNA targets were precipitated and resuspended in hybridization buffer (80% formamide/25 mM sodium citrate) before use. After mixing, the samples were heated 4 minutes at 95°C, then incubated overnight at 42°C. Aliquots (2 μl) of each hybridized sample were treated with 8 μl of RNase solution (15 ng/ml RNase A in 60 mM CsCl/10 mM Tris pH 7.5) for 30 minutes at 37°C. The RNase was then inactivated by addition of 2 μl of 10 mg/ml Proteinase K/2.5 μg/ml yeast RNA and incubation for 15 minutes longer at 37°C. After heating for 3 minutes at 95°C, the samples were loaded onto a 1.5 mm thick 5% polyacrylamide gel containing 8 M urea (*i.e.*, a denaturing polyacrylamide gel) and electrophoresed at 250 volts for $\sim$45 minutes. The gel was exposed to X-ray film overnight with intensifying screen.

Non-isotopic RNase Cleavage Assay

Sense and Antisense transcripts of a 582 bp region of the Factor IX gene and a 969 bp region of the Factor IX gene (coding region and flanking intron) were synthesized from PCR™ templates containing opposable phage promoters. The PCR™ templates were amplified from genomic DNA from hemophilia-B patients and normal controls. Complementary transcripts from patient and control templates were mixed, heated 3 minutes at 95°C, and cooled to room temperature to make the hybridized double-stranded RNA substrates.

Aliquots (4 μl) of the RNA substrates were treated with 16 μl of RNase solution (8 μg/ml RNase A in 0.5M tetramethylammonium chloride (TMAC)/50 mM Tris pH 7.5/0.5 μg/ml ethidium bromide) for 30 minutes at 37°C. Gel loading solution (3 μl) containing 0.5 M TMAC/10 mM Tris 7.5/2 mM EDTA/0.1% bromophenol blue/50% glycerol was added to each sample and the reactions (23 μl) were loaded onto a 2% agarose gel containing 0.5 μg/ml ethidium bromide. The gel was submerged in TBE running buffer and the RNase cleavage products in each reaction were separated by electrophoresis at about 80 volts for ~45 minutes. The gel was transferred to a transilluminator and photographed under ultraviolet light (254 nm) on Polaroid film.

Results

Conventional RNase Mismatch Assay

FIG. 1 shows an example of non-specific background fragments (*i.e.*, fragments common to all lanes, even the no-mismatch controls) in an RNase mismatch assay using radiolabeled probes hybridized to DNA targets of 401 bp comprising about half of exon 8 of the clotting Factor IX gene. The RNase cleavage products were analyzed on a denaturing polyacrylamide gel. Note that the pattern of background fragments is different depending on whether the Sense or the Antisense probe is used.

Non-isotopic RNase Cleavage Assay

FIG. 10A and FIG. 10B shows the results of analyzing the entire Factor IX eighth exon as a single fragment of 582 bp on a native agarose gel, using the non-isotopic RNase cleavage method (the present invention). Note that the non-specific bands are relatively less pronounced (compared to those in FIG. 1), and that the mismatch-specific cleavage products are generally sharper and better resolved on the gel (the better the resolution of the cleavage products, the more accurately the relative position of the mutation within the target region can be mapped).

To determine whether even larger target regions could be screened, the Factor IX target regions from the same genomic DNA samples used for the study in FIG.12A and 12B was reamplified, as part of a larger PCR™ product of 969 bp (by moving the original sense-strand primer several hundred base pairs upstream into the flanking intron), and tested these longer substrates according to the method of the present invention. FIG. 2 shows that the mutations detected in the 582 bp target in FIG.12A and 12B are generally easily detected also in the 969 bp target, although the level of non-specific background signal is somewhat higher. In this study a previously unknown intron polymorphism (mutation) was detected in one of the samples.

In separate blinded screens of 49 genomic DNA samples from Hemophilia B patients and normal controls, 77% of the mutations in exon 8 of Factor IX gene were detected using radiolabeled probes and analyzing the cleavage products on denaturing polyacrylamide gels, while 90% of the mutations were detected using non-isotopic probes (of ~50% greater size) analyzed on native agarose gels, according to the method of this invention. The results of the blinded screens are summarized in Table 2.

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TABLE 2

SUMMARY OF RESULTS OF BLINDED SCREENING OF 49 SAMPLES FOR MUTATIONS IN EXON 8 OF FACTOR IX GENE

		^{32}P-Probes, DNA Targets				NIRCA Method, RNA Targets				
		Sense Probes		Antisense Probes		Sense Probe		Antisense Probe		
Original Sample #	Mutation	Mis-match	Detection	Mis-match	Detection	Mis-match	Detection	Mis-match	Detection	Position of Mutation[1]
2	T→A	rU/dT	ND	rA/dA	⊕	U/U	⊕	A/A	⊕	34004
3	T→C	rU/dG	ND	rA/dC	ND	U/G	±	A/C	⊕	34006
5	G→A	rG/dT	ND	rC/dA	ND	G/U	ND	C/A	ND	34017
6	C→T	rCdA	⊕	rG/dT	ND	C/A	⊕	G/U	⊕	34056
9	C→G	rC/dC	ND	rG/dG	ND	C/C	⊕	G/G	ND	34083
11	C→T	rC/dA	⊕	rG/dT	ND	C/A	⊕	G/U	ND	34083
12	G→T	rG/dA	ND	rC/dT	⊕	G/A	ND	C/U	⊕	34084
15	G→A	rG/dT	ND	rC/dA	ND	G/U	ND	C/A	ND	34084
16	T→A	rU/dT	ND	rA/dA	⊕	U/U	⊕	A/A	⊕	34092
17	A→T	rA/dA	⊕	rU/dT	ND	A/A	⊕	U/U	⊕	34126

TABLE 2 (continued)

		^{32}P-Probes, DNA Targets				NIRCA Method, RNA Targets				
		Sense Probes		Antisense Probes		Sense Probe		Antisense Probe		
Original Sample #	Mutation	Mis-match	Detection	Mis-match	Detection	Mis-match	Detection	Mis-match	Detection	Position of Mutation[1]
18	C→A	rC/dT	⊕	rG/dA	⊕	C/U	⊕	G/A	⊕	34061
20	G→A	rG/dT	ND	rC/dA	ND	G/U	ND	C/A	ND	34012
21	G→C	rG/dG	ND	rC/dC	⊕	G/G	⊕	C/C	ND	34016
23	C→T	rC/dA	ND	rG/dT	ND	C/A	⊕	G/U	ND	34096
24	T→C	rU/dG	ND	rA/dC	ND	U/G	ND	A/C	ND	34097
26	C→T	rC/dA	⊕	rG/dT	ND	C/A	⊕	G/U	ND	34098
27	G→C	rG/dG	ND	rC/dC	⊕	G/G	⊕	C/C	⊕	34099
28	C→G	rC/dC	⊕	rG/dG	⊕	C/C	⊕	G/G	⊕	34105
29	C→A	rC/dT	⊕	rG/dA	⊕	C/U	⊕	G/A	⊕	33893
32	A→T	rA/dA	⊕	rU/dT	ND	A/A	⊕	U/U	ND	33894
33	T→C	rU/dG	ND	rA/dC	ND	U/C	ND	A/C	⊕	33895
35	T→G	rU/dG	⊕	rA/dC	ND	U/G	⊕	A/C	ND	33901

TABLE 2 (continued)

		^{32}P-Probes, DNA Targets				NIRCA Method, RNA Targets				
		Sense Probes		Antisense Probes		Sense Probe		Antisense Probe		
Original Sample #	Mutation	Mis-match	Detection	Mis-match	Detection	Mis-match	Detection	Mis-match	Detection	Position of Mutation[1]
36	T→C	rU/dG	ND	rA/dC	⊕	U/G	ND	A/C	⊕	33910
37	C→A	rC/dT	⊕	rG/dA	⊕	C/U	⊕	G/A	⊕	33938
39	T→G	rU/dC	⊕	rA/dG	⊕	U/C	⊕	A/G	⊕	33946
43	G→T	rG/dA	ND	rC/dT	⊕	G/A	⊕	C/U	⊕	33966
44	C→T	rC/dA	⊕	rG/dT	ND	C/A	⊕	G/U	ND	33973
46	G→T	rG/dA	⊕	rC/dT	⊕	G/A	⊕	C/U	⊕	33952
47	T→G	rU/dC	⊕	rA/dG	ND	U/C	⊕	A/G	±	34117
48	C→T	rC/dA	⊕	rG/dT	ND	C/A	⊕	G/U	ND	34045
⊕ = detected ND = not detected 1) Genbank Sequence: humfixg.pr_gb		Total # Detected (Sense or Antisense): 23 Total # Samples : 49 Total % Detected : 77% Specificity : 86% False positives : 0				Total # Detected (Sense or Antisense) : 27 Total # Samples : 49 Total % Detected : 90% Specificity : 89% False positives : 2				

EXAMPLE 3

Non-Isotopic Screening for *p53* Mutations

Materials and Methods

The Non-isotopic RNase cleavage assay was also used to screen for mutations in exons 5-10 of the *p53* tumor suppressor gene. 802 bp coding regions were screened after amplification from normal and tumor tissue using a 2-step nested RT-PCR™ protocol. The first step (RT-PCR™) consisted of a coupled reverse transcription reaction with AMV reverse transcriptase and ~1-2 pg of total cellular RNA from the clinical samples, followed by PCR™ amplification. AN aliquot of the first step PCR™ was then used as template for amplification of a nested region containing *p53* exons 5-10 plus ~40 bp of flanking exon 4/exon 11 sequences; the T7 and SP6 phage promoters were incorporated into the 5' ends of the primers used in the nested PCR™. The nested PCR™ strategy was able to compensate for a sub-optimal RT-PCR™ step; the yield of primary RT-PCR™ product was too low to visualize by *ethidium bromide* staining in most of the samples, but the secondary (nested) PCR™ product was easily visualized by analyzing ~10% of the reaction on an ethidium bromide-stained agarose gel. Aliquots of the crude nested PCR™ products from experimental samples and a normal control were transcribed, hybridized, and treated with RNase for detection of mismatches as described in the previous examples.

Results

Mutations were detected in two out of seven of the samples amplified from solid tumors; in both cases, cleavage products were seen with both types of RNA/RNA substrate (Wild type control transcript-Sense/Test sample transcript-Antisense and Wild type control transcript-Antisense/Test sample transcript-Sense). This experiment demonstrates the utility of the non-isotopic RNase cleavage assay for mutation detection

in long expressed sequences from autosomal genes in clinical samples. It has also been used to detect new *p53* mutations in targets of 500-600 bp (containing exons 5 and 6 with intervening intron, or exons 8 and 9 plus intron) which were amplified from genomic DNA.

EXAMPLE 4

High Ionic Strength Loading Solution Study

Materials and Methods

Duplicate reactions containing RNase cleavage products from 969 bp substrates were loaded using either 1/6 volume of a standard gel loading solution (10 mM Tris pH 7.5/2 mM EDTA/0.1% bromophenol blue/50% glycerol) (left-hand panel of FIG. 3), or 1/6 volume of the high ionic strength gel loading solution (Final concentration; 0.5 M tetramethylammonium chloride/10 mM Tris pH 7.5/2 mM EDTA/0.1% bromophenol blue/50% glycerol) (right-hand panel of FIG. 3). Materials and methods are as described in the previous examples.

Results

In addition to providing new methods for the analysis of the RNase cleavage products, the present invention also provides new compositions and kits that significantly enhance the resolution of the cleavage products and increase the ability to detect mutations with the relatively low-resolution agarose gels. The novel composition is a high ionic strength gel-loading solution that contains, in addition to the standard components, such as glycerol and bromophenol blue, a concentration of salts sufficient to retard the mobility of the double-stranded RNA fragments and cause them to focus into sharp bands. Suitable salts are sodium chloride and tetramethylammonium chloride (TMAC).

FIG. 3 shows the results of using the standard agarose gel-loading solution (50% glycerol/10 mM Tris 7.5/0.1% bromophenol blue/2 mM EDTA) and the new gel-loading solution in the analysis of the 969 bp targets used in the study shown in FIG. 2, Example 2. The cleavage products loaded in the standard solution (lanes 1 → 7) migrate as wide, diffuse bands which are difficult to visualize against the background of non-specific ethidium-stained material. In contrast, the products of duplicate reactions loaded with the high ionic strength gel-loading solution (lanes 8 → 14) (3 M TMAC/10 mM Tris 7.5/2 mM EDTA/0.1% bromophenol blue/50% glycerol, to give a final TMAC concentration of 0.5M) migrate as sharp, well-resolved bands that are easily distinguished from the background signal in the no-mismatch control lane (lanes 1 and 8 are no-mismatch control lanes).

EXAMPLE 5

A Kit for Use in Detecting Single Base Changes in a Nucleic Acid Sequence

Further aspects of the invention concern kits for use in RNase protection assays. Such kits will generally have as one component an enzyme or enzymes capable of specific cleavages of double stranded RNA and capable of detecting single base mismatches in an RNA/RNA duplex. Most preferably, the enzyme will be RNase A or a mixture of RNase A and RNase B.

The enzyme or enzymes may be provided in lyophilized, powdered form to be stored in a freezer at about -20°C to about -80°C and to be mixed with solvents in the indicated concentrations just prior to use, or alternatively, the enzyme or enzymes may be provided in solution in an appropriate buffer and possibly glycerol solution. All of the said solvents and buffers may or may not be provided with the kit.

The kits will generally also contain a high ionic strength gel-loading solution for use in significantly enhancing the resolution of the RNase cleavage products. Such a solution will contain, in addition to the standard components, a concentration of a salt sufficient to retard and to focus double-stranded RNA fragments into sharp bands. Suitable salts are sodium chloride and tetramethylammonium chloride (TMAC). An example of a high ionic strength gel-loading solution is 3 M TMAC, 10 mM Tris 7.5, 2 mM EDTA, 0.1% bromophenol blue, 50% glycerol. The buffer may be provided as a premixed solution or as various aliquots of components for forming such a solution.

Other components of the kit may include, but are not limited to, components for preparing a non-denaturing gel, a solution of enzyme buffer and/or a solution of NaCl. Sterile, nuclease free, purified water may also be included in the kit. The kit may also contain a control RNA template(s) with and/or without a mismatch with a complementary RNA probe. The RNA molecules will be provided in solution or in lyophilized form and would be stored at 4°C or alternatively could be stored at -20°C to -80°C.

The kit may also contain PCR™ primers to be used to amplify the test sample. The primers will preferably be comprised of phage promoter sequences to be used in the transcription of the PCR™ products. The primers may also comprise sequences complementary to the regions of test sample being amplified. The phage promoter sequences are appended to the 5' ends of the test gene-specific sequences.

A specific example of a kit in accordance with the invention is that which includes a 10X transcription buffer and rNTP mix; SP6 and T7 RNA polymerases (20 Units/ml each); a hybridization solution; an RNase stock solution and digestion buffer; the afore-mentioned improved gel loading solution; RNase-free dH_2O; a wild type and mutant control template, *e.g.*, for the *p53* gene; and control probes (amplimers) with phage promoters.

EXAMPLE 6

Point Mutation Analysis of Genes

The compositions, techniques and kits of the present invention may be used to detect mutations in virtually any nucleic acid segment. Thus, this improved technique will be useful in screening genes for mutations associated with many human cancers, such as, for example, mutations in *p53*, *ras*, *neu*, *myc*, *abl*, and other disease-associated mutations in these or any other genes, including HIV reverse transcriptase and β globin. Other genes that may be screened for mutations include, but are not limited to APC, which is thought to be associated with early events in the colon cancer pathway, NF1, the gene implicated in neurofibromatosis (NF) and BRCA1, recently implicated in familial breast cancer etiology. The ability to accurately detect mutations, including single base mutations in these and other regulatory and structural genes will be useful in detection, therapy and counseling associated with a wide variety of genetic disorders.

In addition to the clinical and genetic counseling applications, the present invention will be useful as a rapid, accurate and economical screening method to detect mutations in studies of protein structure and folding, enzymatic mechanisms, RNA structure in population genetic studies, and in antibody-antigen recognition and binding studies involving proteins, DNA and RNA. This would include, but is not limited to mutations created by site-directed mutagenesis, UV irradiation, chemical mutagenesis, random primer mutagenesis and mutations created by PCR™ methods.

EXAMPLE 7

RNase Cleavage Assay for the Detection of Mutations

In performing an RNase cleavage assay of base pair mismatches, the inventor currently prefers to use certain compositions and steps, which are set forth in the present example.

Both strands (sense and antisense) of the test and control target regions are transcribed into RNA. Preferably, transcription reactions contain mass amounts of unlabeled ribonucleotides sufficient to generate several micrograms of transcript at a concentration of about 100-500 μg/ml. Typical *in vitro* transcription reactions contain:

A. 2 μl PCR™ template with opposable promoters, for example, T7 and SP6 promoters;

B. 2 μl of a 5x buffer of buffer containing salts, spermidine, and buffering and reducing agents. The 5x transcription buffer contains:

(a) 200 mM Tris-Cl pH 8;

(b) 40 mM $MgCl_2$;

(c) 10 mM spermidine; and

(d) 250 mM NaCl as described in *Current Protocols in Molec. Biology* (1) 4.7.5.

C. 2 μl of a mixture containing all 4 ribonucleotide triphosphates, each at a concentration of 2.5 mM;

D. 4 μl distilled H_2O; and

E. 1 μl of 20 units/μl RNA polymerase, eg. T7 or SP6 RNA Polymerase.

The template for the *in vitro* transcription reaction generally contains amplified products from test or wildtype control target regions. Alternatively, the test and wildtype control target regions may both exist in a single sample amplified from a heterozygous source. The RNA polymerase used may be a mixture of polymerases, for example T7 and SP6.

The reactions are incubated and terminated according to standard procedures. Preferably, they are terminated by heating in an equal volume of buffer containing a high formamide and low salt concentration, for example, 80% formamide/25 mM sodium citrate at pH 6.8.

The sense and antisense products of the *in vitro* transcription reactions are mixed such that complementary transcripts derived from wildtype control and test target regions are able to hybridize and form double-stranded nucleic acid molecules. The complementary sense and antisense transcripts derived from the wildtype control template are also mixed and hybridized. Typically, at least 2 μl of each transcription reaction is mixed with 2 μl of its complement, heated briefly (~3 minutes) at 80-100°C, and allowed to cool to ambient temperature over a period of several minutes. When the template for the transcription reaction included a mixture of control and test target templates, such that both test and wildtype complementary transcripts were produced simultaneously, in the *in vitro* transcripts reaction, this mixing step may not be required.

The hybridized wildtype control and test transcripts (RNA test duplexes), and also the hybridized complementary wildtype control transcripts (RNA control duplexes), are treated with ribonuclease(s) or other enzymes or agents such that cleavage of target duplexes containing base pair mismatches takes place to generate products which are altered in size compared to the control duplex treated in the same way. Preferably, the volume of solution (for example nuclease solution) added to the RNA duplexes should be sufficiently small that the final reaction products can be analyzed directly on a gel. For example, 16 μl of a solution containing a nuclease capable of cleaving base pair mismatches is typically added to 4 μl of an RNA duplex, mixed, and incubated at 37°C for 20 to 60 minutes.

The solution added to the RNA/RNA or RNA/DNA duplex also may contain 32 mg/ml tryptone, 100 μg/ml ethidium bromide, and one or more of the following ribonucleases: RNase I, at about 100 to about 300 units/ml; RNase A, at 0.3 μg/ml; RNase T1, at about 64,000-160,000 units/ml (equivalent to about 100-250 μg/ml). With the addition of 4 μl the test duplex, the final concentration will become about 80 μg/ml ethidium bromide and about 25 mg/ml tryptone.

Alternatively, the solution added to the RNA/RNA or RNA/DNA duplex may contain 25 μg/ml ethidium bromide, 25 mg/ml tryptone, 85 mM NaCl, and one or more of the following ribonucleases: RNase I, at 500-1000 units/ml; RNase A, at 0.1-0.5 μg/ml; RNase T1, at 64,000-160,000 units/ml. If the ribonuclease used is RNase T1, the tryptone may be omitted from the solution.

The reaction products are analyzed in the double stranded state, to detect size differences in the test duplexes compared to the control duplex. Typically, a small amount (~1/6-1/10 volume) of a dense, loading solution is added to all samples, which are then loaded directly, without heating, onto a non-denaturing gel, typically 2% agarose in 1X TBE. 1X TBE is Tris-borate-EDTA electrophoresis buffer; 90 mM Tris/borate. 2mM EDTA containing 0.5 μg/ml ethidium bromide. The nucleic acid fragments in each sample are separated according to size by running the gel, *i.e.*, by electrophoresis at 80 volts for 30 minutes in a buffer containing 1X TBE.

The fragments are visualized by staining with a dye that binds to double-stranded nucleic acid, such as ethidium bromide. The ethidium dye may be added to the sample, the gel, and/or the buffer, or the finished gel may be soaked in solution containing ethidium bromide. The dense loading solution may also contain ingredients such as tetramethylammonium chloride or ethidium bromide that cause the nucleic acid fragments to migrate as sharp bands, rather than diffuse bands, which would be difficult to analyze. The gels are illuminated with ultraviolet light to visualize the ethidium-stained nucleic acid fragments. When the RNase digestion buffer contains ethidium (as in the present invention), it is not necessary to add it to the gel or the electrophoresis buffer.

Test samples containing nucleic acid fragments which are not seen in the no-mismatch control reaction are scored as positive for containing base pair mismatches, which result from mutations in the test target region being screened. The test samples may be derived from nucleic acid targets amplified from chromosomal DNA (*i.e.*, genomic

DNA) from prokaryotic or eukaryotic sources, including plant, animal, bacterial, fungal, or protozoan genomes, or from mitochondrial DNA from plant or animal or fungal eukaryotic sources.

EXAMPLE 8

Cleavage of Mismatches Using Prior Art and Current Invention Compositions for RNase I Digestion Buffer

This experiment describes the cleavage and detection of mutations using the inventive buffers disclosed in this patent as compared with buffers that have previously been reported. Double-stranded RNA targets were prepared by *in vitro* transcription of PCR™ products containing opposable T7 and SP6 promoters, according to the basic NIRCA™ method. Targets were 582 bp regions of the clotting Factor IX gene, amplified from genomic DNA from Hemophilia B patients having known mutations in exon 8.

FIG. 4 shows the results of comparing the detection of mutations by buffers of the present invention and the prior art. The mismatches resulting from hybridization of patient sense strand and wildtype antisense strand transcripts are shown above the lanes. Samples in the left side of FIG. 4 (lanes 1-15) were treated with 2.6 units/reaction (0.165 units/μl) of RNase I in a buffer containing 32 grams/liter tryptone, 100 μg/ml ethidium bromide, and 50 μg/ml carbenicillin (to prevent bacterial growth); this is one of the preferred buffer compositions of the present invention. Duplicate samples in the right side of FIG. 4 (lanes 16-30) were treated with 53 units/reaction (3.3 units/μl) of RNase I in a buffer containing 10 mM Tris pH 7.5, 5 mM EDTA, and 200 mM sodium acetate; this is the buffer composition given in the prior art (Promega, Inc.). All samples were incubated for 45 minutes at 37°C, and analyzed on a 2% agarose gel according to the standard NIRCA™ procedure.

Many mismatches cleaved in lanes 1-15 employing the buffer of this invention were not cleaved or were less completely cleaved, as compared to the same samples in lanes 16-30 by the prior art buffer, despite the fact that 20-fold greater amount of RNase I was used to treat the samples in lanes 16-30.

EXAMPLE 9

Cleavage of Mismatches Using an RNase T1 Digestion Buffer

This example details the detection of mutations employing RNase T1 buffers of the present invention. Double-stranded RNA targets containing mismatches due to point mutations in exon 8 of the Factor IX gene were prepared as described in Example 7.

The results are illustrated in FIG. 5. Mismatches in each sample are shown above the lanes; the lanes labeled "wt" contain the wildtype (no-mismatch) control sample. Samples were treated with RNase T1 at a concentration of 250 μg/ml for 45 minutes at 37°C and analyzed on 2% agarose gels according to the standard NIRCA^{TM} protocol. The digestion buffer used for the samples shown on the left side of FIG. 5 (lanes 1-15) contained 32 grams/liter tryptone, 100 μg/ml ethidium bromide, and 50 μg/ml carbenicillin; this is one of the preferred buffer compositions of the present invention. The digestion buffer used for the duplicate samples shown on the right side of FIG. 5 (lanes 16-30) contained 10 mM Tris pH 7.5 and 300 mM sodium chloride; this is the composition of the standard buffer used for RNase A cleavage in the prior art (Winter *et al.*, 1985), except that the EDTA was omitted. (Since RNase T1 has not previously been widely used for mismatch cleavage, there is no prior art for an RNase T1-mismatch digestion buffer.) Many mismatches cleaved in lanes 1-15 were not cleaved or were less completely cleaved, as compared to the same samples in lanes 16-30.

EXAMPLE 10

Cleavage of Mismatches Using a Digestion Buffer with Calcium

This example teaches the cleavage and detection of mutations employing calcium instead of tryptone as the basepair mismatch cleavage enhancing agent in the digestion buffer. Double-stranded RNA targets containing mismatches due to point mutations in exon 8 of the Factor IX gene were prepared as described in Example 7. Replicate panels of 6 targets were treated with RNase I (0.165 units/μl; 2.6 units/reaction) in one of five different digestion buffers and analyzed on a 2% agarose gel according to the standard NIRCA^{TM} protocol.

The results are depicted in FIG. 6. Mismatches in each sample are shown above the lanes. Lanes 1-6 show the results of employing a preferred digestion buffer of this invention that contained 32 grams/liter tryptone and 100 μg/ml ethidium bromide. In this case, cleavage products were generated in all samples.

In lanes 7-12, the results are illustrated for a digestion buffer that contained 10 mM Tris pH 7.5, 40 mM sodium chloride, and 100 μg/ml ethidium bromide. Note that no cleavage products were generated.

In lanes 13-18, another preferred digestion buffer of this invention was employed. This buffer contained 10 mM Tris pH 7.5, 40 mM sodium chloride, 100 μg/ml ethidium bromide, and 1.5 mM calcium chloride (the same as that used in Lanes 7-12, except that calcium chloride was added). Cleavage products were again seen in all samples. Lanes 19-24 also employed a digestion buffer that was the same as in lanes 7-12, except that calcium chloride was added to 2.5 mM. Cleavage products were again seen in all samples. Lanes 25-30 also used a digestion buffer that was the same as in Lanes 7-12,

except that calcium chloride was added to 4 mM. Cleavage products were also seen in all samples.

EXAMPLE 11

Cleavage of Mismatches in the Presence of Acridine Orange

This example details the cleavage and detection of mismatches by digestion buffers containing acridine orange. Double-stranded RNA targets containing mismatches due to point mutations in exon 8 of the Factor IX gene were prepared as described in Example 7. Replicate panels of 6 targets were treated with RNase I (1 unit/μl; 16 units/reaction) in one of five different digestion buffers and analyzed on a 2% agarose gel according to the standard NIRCA^{TM} protocol.

FIG. 7 depicts the results of this example. The mismatches in each sample are shown above the lanes; the lanes labeled "wt" contain the wildtype (no-mismatch) control sample. The basal digestion buffer used for all samples contained 32 grams/liter tryptone and 85 mM sodium chloride. In addition, intercalating agents (acridine orange or ethidium bromide) were added to some samples as follows: Lanes 1-6: 5 μg/ml acridine orange; Lanes 7-12: 10 μg/ml acridine orange; Lanes 13-18: 20 μg/ml acridine orange; Lanes 19-24: no intercalating agent; Lanes 25-30: 10 μg/ml ethidium bromide. Cleavage of most mismatches was improved in the presence of an intercalating agent. The cleavage products migrate as bands that are more diffused in the presence of acridine orange, compared to their migration in the presence of ethidium bromide.

EXAMPLE 12

The Interrelationship of Tryptone and Calcium and the Metal Chelators EDTA and EGTA

This example describes the interrelationship between tryptone and calcium in the practice of the present invention. For example, the beneficial effects of tryptone for mismatch cleavage can be duplicated by calcium and are abolished by EDTA and EGTA. Double-stranded RNA targets containing mismatches due to point mutations in exon 8 of the Factor IX gene were prepared as described in Example 7.

The results are illustrated in FIG. 8A, FIG. 8B, FIG. 8C, FIG. 8D, and FIG. 8E. Each target was treated with RNase I (0.165 units/μl) under 10 different RNase digestion buffer conditions, which correspond to the lane numbers in FIG. 8A, FIG. 8B, FIG. 8C, FIG. 8D, and FIG. 8E: in conditions 1, 8, 9, and 10, the digestion buffer contained 32 grams/liter tryptone and 100 μg/ml ethidium bromide ("tryptone buffer"); in conditions 2, 3, 4, 5, 6, and 7, the digestion buffer contained 10 mM Tris pH 7.5, 40 mM sodium chloride, and 100 μg/ml ethidium bromide ("defined buffer"). Some reactions contained calcium (added as calcium chloride) as follows: condition 3: 0.1 mM calcium; condition 4: 1 mM calcium; condition 5: 2.5 mM calcium; condition 6: 5 mM calcium; condition 7: 10 mM calcium. Some reactions contained a divalent cation chelating agent as follows: condition 8: 1 mM EDTA; condition 9: 3 mM EDTA; condition 10: 1mM EGTA. The mismatch in each target is indicated above each figure.

The presence of cleavage products was detected when using the tryptone buffer (condition 1), and the cleavage products were absent when using the defined buffer without calcium (condition 2). Cleavage products were seen in the defined buffer when calcium is added to 1 mM (condition 4), but not when calcium was added to only 0.1 mM (condition 3). Addition of calcium to levels above 1 mM resulted in overdigestion of the target duplex (conditions 5, 6, and 7). When the tryptone buffer was used, addition

of EDTA to 1 mM or 3 mM (conditions 8 and 9), or addition of EGTA to 1 mM (condition 10), prevented the mismatch from being cleaved.

EXAMPLE 13

Cleavage of Mismatches in *p53* Targets by RNase A, RNase I, and RNase T1

This example describes the cleavage of mismatches in *p53* targets by digestion buffers of the present invention and RNase A, RNase I, or RNase T1. *p53* is a tumor suppressor included in cancer. The ability to detect mutations in the *p53* gene allows one's cancer-susceptibility to be determined. This example therefore demonstrates the utility of the present invention in a clinical environment. Double-stranded RNA targets were prepared by *in vitro* transcription of PCR™ products containing opposable T7 and SP6 promoters, according to the basic NIRCA™ method. Targets were 781 bp coding regions (exons 4-10) of the *p53* tumor suppressor gene, amplified by RT-PCR™ from total RNA samples isolated from unselected breast tumors. The RNase digestion buffer for all samples contained 32 grams/liter tryptone and 100 μg/ml ethidium bromide.

The results are shown in FIG. 9A, FIG. 9B and FIG. 9C. The RNases used to treat the samples were as follows: FIG. 9A: RNase I, 0.165 units/μl; FIG. 9B: RNase T1, 250 μg/ml; FIG. 9C: RNase A, 0.3 μg/ml. The sample numbers are shown above the lanes; samples designated "T" were prepared by hybridizing experimental Antisense strands with wildtype Sense-strand transcripts; samples designated "S" were prepared by hybridizing experimental sense strands with wildtype antisense strands. Lanes labeled "wt" contain the wildtype (no-mismatch) control sample.

Some samples showed cleavage products with more than one RNase. The cleavage products were due to putative *p53* point mutations in the experimental samples; the nature of the mutations is presently being determined by direct sequencing of PCR™

products. Some samples did not show cleavage products with any of the three RNases; these are putatively negative for *p53* point mutations.

EXAMPLE 14

Cleavage of Mismatches in Large Panel of Homozygous and Heterozygous Samples with Factor IX Mutations.

This example details the cleavage and detection of mismatches with digestion buffers of the present invention in large panel homozygous and heterozygous samples with Factor IX mutations. Double-stranded RNA targets containing mismatches due to point mutations in exon 8 of the Factor IX gene were prepared from genomic DNA isolated from Hemophilia B patients and heterozygous carriers, as described in Example 7.

The results are illustrated in FIG. 10A and FIG. 10B. The mismatches in each sample are shown above the lanes. Samples from heterozygous carriers are designated with a dot. Lanes designated "wt" contain wildtype (no-mismatch) control sample. The RNase digestion buffer used for all samples contained 32 grams/liter tryptone and 100 *μg/ml* ethidium bromide. Lanes marked "mw" contained molecular weight markers (Sau 3A restriction fragments of plasmid pUC19); the sizes of the two largest mw fragments were 955 bp and 585 bp. The RNase used to treat each group of samples is indicated below the figure.

Specific cleavage products were generated in most but not all samples, by at least one (and usually more than one) of the three RNases. Samples that were negative with all three RNases were positive with at least one RNase when the reciprocal mismatch was tested.

EXAMPLE 15

Fractionation of the Protein Mixture to Identify the Component(s) Active in Enhancing RNase Cleavage of Mismatches

This example describes how one might identify the various active components that result in enhancing the specific cleavage of base pair mismatches.

In order to identify the active component(s) in a protein mixture, such as tryptone, that are shown to be effective in improving mismatch detection in the RNase assay, it is contemplated that one may initially use ion exchange HPLC (high-performance liquid chromatography). Both cation and anion exchange resins may be used. Individual column fractions may then be assayed for their ability to replace tryptone in mismatch cleavage assays. Those fractions with cleavage enhancing activity may be scanned spectroscopically, in both visible and ultraviolet wavelengths, in order to identify relevant absorbance peaks. If fractions with mismatch cleavage enhancing activity do not correlate with any specific peaks, the fractions may be analyzed by flame spectroscopy for the presence of metals or other inorganic ions. Once the active substance has been identified, if it is not a metal ion, its identity may be determined by infrared spectroscopy. The abundance and low cost of the starting material, tryptone, will facilitate the isolation and identification of the active component.

As an alternative to the above scheme for identifying the active component, one may select components from the list of ingredients found in tryptone (see attached detailed biochemical analysis provided by Difco laboratories, the company which manufactures tryptone), and test individual components, such as vitamins, co-factors, and ions, for mismatch cleavage enhancing activity.

• • •

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS:

1. A method for cleaving a double-stranded nucleic acid molecule that contains a base pair mismatch, comprising the steps of:

(a) obtaining a test nucleic acid duplex;

(b) treating said test nucleic acid duplex with a ribonuclease composition capable of cleaving double-stranded nucleic acid molecules containing base pair mismatches under conditions effective to allow the formation of double stranded cleavage products; and

(c) separating said cleavage products under conditions that allow the cleavage products to remain double-stranded.

2. The method of claim 1, wherein said test nucleic acid duplex is an RNA duplex comprising an RNA test sample and an RNA probe of known sequence.

3. The method of claim 2, wherein step (a) comprises obtaining a single-stranded RNA test sample and contacting said test sample with a single-stranded RNA probe under conditions effective to form a test duplex.

4. The method of claim 2, wherein one component of said test RNA duplex comprises a label that is either a non-radioactive or a radioactive label.

5. The method of claim 4, wherein the label is nonradioactive and the label is fluorescein or rhodamine.

6. The method of claim 4, wherein said label is a radioactive label.

7. The method of any one of claims 2 to 6, wherein said RNA test sample is prepared by transcription of a recombinant plasmid.

8. The method of any one of claims 2 to 6, wherein said RNA test sample is prepared by transcription of a PCR product.

9. The method of any one of claims 2 to 8, wherein said RNA test sample is prepared by transcription of a recombinant plasmid or PCR product that contains opposable promoters.

10. The method of claim 9, wherein said recombinant plasmid or PCR product contains an SP6, T7 or T3 promoter.

11. The method of claim 9, wherein said opposable promoters are the same, allowing transcription by a single RNA polymerase.

12. The method of any one of claims 2 to 11, wherein said RNA probe is prepared by transcription of a PCR product.

13. The method of claim 12, wherein said RNA test sample and said RNA probe are prepared by transcription of a PCR product.

14. The method of any one of claims 2 to 13, wherein said RNA test sample is purified from a cellular extract.

15. The method of any one of claims 2 to 14, wherein said RNA test sample is obtained from a biological sample from an animal or patient suspected of having a disease associated with a genetic mutation.

16. The method of any one of claims 2 to 15, wherein said RNA test sample is obtained from a biological sample from an animal or patient suspected of having cancer.

17. The method of any one of claims 2 to 16, wherein said RNA test sample is associated with a solid support prior to treatment with said ribonuclease composition.

18. The method of any one of claims 1 to 17, wherein said ribonuclease composition comprises RNase I, RNase I*, RNase YI* from yeast, RNase T1, RNase A, modified RNase A, RNase B or a mixture of RNase A and RNase B.

19. The method of claim 18, wherein said ribonuclease composition comprises two or more RNase enzymes.

20. The method of claim 18, wherein said ribonuclease composition comprises RNase I.

21. The method of any one of claims 1 to 20, wherein said ribonuclease composition comprises an RNase enzyme in a concentration of between about 0.01 μg/ml and about 500 μg/ml.

22. The method of any one of claims 1 to 21, wherein said cleavage products are separated using non-denaturing gel electrophoresis.

23. The method of claim 22, wherein said cleavage products are separated using a non-denaturing agarose gel.

24. The method of claim 22, wherein said cleavage products are separated using a non-denaturing polyacrylamide gel.

25. The method of any one of claims 22 to 24, wherein said cleavage products are admixed with a high ionic strength loading solution to form a loading sample prior to separation by non-denaturing gel electrophoresis.

26. The method of claim 25, wherein said loading solution comprises a salt in a concentration sufficient to provide a final salt concentration in each loading sample of at least about 0.5 M.

27. The method of claim 25 or 26, wherein said loading solution comprises a tetramethylalkyl salt.

28. The method of claim 27, wherein said loading solution comprises tetramethylammonium chloride.

29. The method of claim 25 or 26, wherein said loading solution comprises NaCl.

30. The method of any one of claims 22 to 29, wherein said cleavage products are contacted with an agent that causes the cleavage products to fluoresce.

31. The method of claim 30, wherein said cleavage products are contacted with ethidium bromide.

32. The method of claim 30 or 31, wherein said agent is incorporated into said non-denaturing gel.

33. The method of claim 30 or 31, wherein said agent is incorporated into electrophoresis buffer.

34. The method of claim 30 or 31, wherein said agent is admixed with sample prior to electrophoresis.

35. The method of any one of claims 22 to 29, wherein said cleavage products are analyzed by silver staining.

36. The method of any one of claims 1 to 21, wherein said cleavage products are separated using non-denaturing HPLC or capillary electrophoresis.

37. The method of any one of claims 1 to 36, wherein the separated cleavage products are analyzed using an automated device.

38. The method of any one of claims 1 to 37, wherein said ribonuclease composition comprises:

(a) an RNase enzyme;

(b) a nucleic acid intercalating agent; and

(c) an RNase mismatch cleavage activity enhancing agent comprising a protein mixture, a divalent cation or both.

39. The method of claim 38, wherein said nucleic acid intercalating agent is ethidium bromide.

40. The method of claim 38 or 39, wherein said nucleic acid intercalating agent is present in said ribonuclease composition at a concentration of between about 1 μg/ml and about 1000 μg/ml.

41. The method of any one of claims 38 to 40, wherein said RNase mismatch cleavage activity enhancing agent comprises a digested protein mixture.

42. The method of claim 41, wherein said digested protein mixture comprises tryptone, peptone, casamino acids, N-Z amine, yeast extract, an acid or enzymatic hydrolysate of casein, an acid or enzymatic hydrolysate of soy bean, an acid or enzymatic hydrolysate of meat protein, an acid or enzymatic hydrolysate of mixed protein or brain-heart infusion.

43. The method of claim 42, wherein said digested protein mixture comprises an acid or enzymatic hydrolysate of casein.

44. The method of claim 42, wherein said digested protein mixture comprises tryptone.

45. The method of any one of claims 41 to 44, wherein said digested protein mixture is present in said ribonuclease composition at a concentration of between about 0.2 mg/ml and about 100 mg/ml.

46. The method of claim 42, wherein said ribonuclease composition comprises a digestion buffer comprising between about 10 mg/ml and about 40 mg/ml tryptone and between about 10 μg/ml and about 200 μg/ml ethidium bromide.

47. The method of any one of claims 38 to 46, wherein said RNase mismatch cleavage activity enhancing agent comprises a divalent cation.

48. The method of claim 47, wherein said RNase mismatch cleavage activity enhancing agent comprises a Ca^{++} cation.

49. The method of claim 46 or 47, wherein said divalent cation is present in said ribonuclease composition at a concentration of between about 0.1 mM and about 100 mM.

50. The method of any one of claims 38 to 49, wherein said ribonuclease composition further comprises a monovalent cation.

51. The method of claim 50, wherein said ribonuclease composition comprises NaCl at a concentration of between about 5 mM and about 300 mM.

52. The method of any one of claims 1 to 51, further defined as a method for identifying a mutation in a gene, said method comprising the steps of:

(a) obtaining a test nucleic acid duplex comprising a test nucleic acid sample from the gene to be tested in combination with an RNA probe having a known sequence;

(b) obtaining a control nucleic acid duplex containing a control nucleic acid sample having a known wild type sequence from said gene in combination with said same RNA probe;

(c) performing parallel digestions of said test nucleic acid duplex and said control nucleic acid duplex under conditions effective to allow the formation of cleavage products from any nucleic acid duplex that contains a base pair mismatch;

(d) separating any cleavage products from each parallel digestion under conditions that allow the cleavage products to remain double-stranded; and

(e) comparing any separated cleavage products from said test nucleic acid duplex and said control nucleic acid duplex, wherein a difference in size of any cleavage products from said test nucleic acid duplex and cleavage products from said control nucleic acid duplex is indicative of the presence of a mutation in said gene.

53. The method of claim 52, wherein said RNA probe has a sequence corresponding to a sequence of the wild type gene and forms a duplex with said control nucleic acid sample that does not contain a mismatch.

54. The method of claim 52, wherein said RNA probe has a sequence corresponding to a sequence of the mutant gene and forms a duplex with said control sample that does contain a mismatch.

55. The method of any one of claims 52 to 54, wherein said test nucleic acid sample is obtained from an animal or patient suspected of having a disease associated with a point mutation.

56. The method of any one of claims 52 to 55, wherein said test nucleic acid sample is obtained from an animal or patient suspected of having cancer.

57. A reaction mixture comprising:

(a) an RNase enzyme;

(b) a nucleic acid intercalating agent; and

(c) an RNase mismatch cleavage activity enhancing agent comprising a protein mixture, a divalent cation, or both.

58. The reaction mixture of claim 57, wherein said reaction mixture comprises RNase I, RNase I*, RNase YI* from yeast, RNase T1, RNase A, modified RNase A, RNase B or a mixture of RNase A and RNase B.

59. The reaction mixture of claim 58, wherein said reaction mixture comprises two or more RNase enzymes.

60. The reaction mixture of claim 58, wherein said reaction mixture comprises RNase I.

61. The reaction mixture of any of claims 57-60, wherein said RNase enzyme is present in a concentration of between about 0.01 μg/ml and about 500 μg/ml.

62. The reaction mixture of any of claims 57-61, wherein said nucleic acid intercalating agent is ethidium bromide.

63. The reaction mixture of any of claims 57-62, wherein said nucleic acid intercalating agent is present in a concentration of between about 1 μg/ml and about 1000 μg/ml.

64. The reaction mixture of any of claims 57-63, wherein said RNase mismatch cleavage activity enhancing agent comprises a digested protein mixture.

65. The reaction mixture of claim 64, wherein said digested protein mixture comprises tryptone, peptone, casamino acids, N-Z amine, yeast extract, an acid or enzymatic hydrolysate of casein, an acid or enzymatic hydrolysate of soy bean, an acid or enzymatic hydrolysate of meat protein, an acid or enzymatic hydrolysate of mixed protein or brain-heart infusion.

66. The reaction mixture of claim 65, wherein said digested protein mixture comprises an acid or enzymatic hydrolysate of casein.

67. The reaction mixture of claim 66, wherein said digested protein mixture comprises tryptone.

68. The reaction mixture of any one of claims 64 to 67 wherein said digested protein mixture is present in a concentration of between about 0.2 mg/ml and about 100 mg/ml.

69. The reaction mixture of claim 65, wherein said reaction mixture comprises between about 10 mg/ml and about 40 mg/ml tryptone and between about 10 μg/ml and about 200 μg/ml ethidium bromide.

70. The reaction mixture of any of claims 57-69, wherein said RNase mismatch cleavage activity enhancing agent comprises a divalent cation.

71. The reaction mixture of claim 70, wherein said divalent cation is a Ca^{++} cation.

72. The reaction mixture of claim 70 or 71, wherein said divalent cation is present in a concentration of between about 0.1 mM and about 100 mM.

73. The reaction mixture of claims 57-72, wherein said reaction mixture further comprises a monovalent cation.

74. The reaction mixture of claim 73, wherein said reaction mixture further comprises NaCl.

75. The reaction mixture of claim 74, wherein said NaCl is present at a concentration of between about 5 mM and about 300 mM.

76. The reaction mixture of any of claims 57-75, wherein said reaction mixture further comprises nucleic acid.

77. The reaction mixture of claim 76, wherein said nucleic acid is double stranded nucleic acid.

78. The reaction mixture of claim 77, wherein said double-stranded nucleic acid is double-stranded RNA.

79. A reaction mixture of any of claims 57-78, for use in cleaving RNA.

80. A reaction mixture according to any of claims 57-79, for use in detecting a base pair mismatch in a double stranded nucleic acid.

81. A nucleic acid digestion buffer comprising a digested protein mixture.

82. The nucleic acid digestion buffer of claim 81, wherein said digested protein mixture comprises an acid or enzymatic hydrolysate of casein.

83. The nucleic acid digestion buffer of claim 81 or 82, wherein said digested protein mixture comprises tryptone.

84. The nucleic acid digestion buffer of claim 83, wherein said digestion buffer comprises between about 10 mg/ml and about 40 mg/ml tryptone and between about 10 μg/ml and about 200 μg/ml ethidium bromide.

85. The nucleic acid digestion buffer of any one of claims 81 to 84, wherein said digestion buffer further comprises an intercalating agent.

86. The nucleic acid digestion buffer of claim 85, wherein said intercalating agent is ethidium bromide.

87. The nucleic acid digestion buffer of any one of claims 81 to 86, wherein said digestion buffer further comprises at least one RNase enzyme.

88. The nucleic acid digestion buffer of claim 87, wherein said RNase enzyme is RNase I.

89. A nucleic acid digestion buffer according to any one of claims 81 to 88, for use as an RNA digestion buffer.

90. A nucleic acid digestion buffer according to any one of claims 81 to 89, for use as a buffer in a reaction for detecting base pair mismatches in double stranded nucleic acids.

91. A kit for use in conducting an RNase mismatch cleavage assay, the kit comprising, in a suitably aliquoted form, an RNase enzyme and a means of generating RNA.

92. The kit of claim 91, wherein said kit comprises, in a suitably aliquoted form, an RNA digestion buffer comprising a protein mixture, a divalent cation or both, a means of generating RNA and a means of cleaving RNA.

93. The kit of claim 91 or 92, wherein said kit further comprises a nucleic acid intercalating agent.

94. The kit of claim 92 or 93, wherein said RNA digestion buffer comprises a digested protein mixture.

95. The kit of any one of claims 92 to 94, wherein said RNA digestion buffer comprises a divalent cation.

96. The kit of any one of claims 91 to 95, wherein said means of generating RNA comprises a first and second PCR primer, each primer including a promoter sequence and a nucleotide sequence from opposite strands of spatially separated regions of the same gene, and a RNA polymerase interactive with said promoter sequences.

97. The kit of claim 96, wherein said first and second PCR primers include the same promoter sequence and wherein said kit comprises an RNA polymerase interactive with said promoter sequence.

98. The kit of claim 96, wherein said first and second PCR primers include distinct, first and second promoter sequences and wherein said kit comprises a first RNA polymerase interactive with said first promoter sequence and a second RNA polymerase interactive with said second promoter sequence.

99. The kit of any one of claims 96 to 98, wherein said means of generating RNA further comprises a transcription buffer and a nucleotide solution.

100. The kit of any one of claims 96 to 99, wherein said means of generating RNA further comprises a template gene sequence having the wild type sequence of said gene and a template gene sequence having the mutant sequence of said gene.

101. The kit of any one of claims 96 to 100, wherein said first and second PCR primers include reverse orientation gene sequences from spatially separated regions of the p53 gene.

102. The kit of any one of claims 91 to 101, wherein said kit further comprises an RNase I enzyme.

103. The kit of any one of claims 91 to 102, wherein said kit further comprises an RNA hybridization buffer.

104. The kit of any one of claims 91 to 103, wherein said kit further comprises a high ionic strength gel loading solution.

105. A high ionic strength gel loading solution, comprising a concentration of a salt sufficient to provide a final salt concentration of at least about 0.5 M upon admixing with a sample.

106. The high ionic strength gel loading solution of claim 105, wherein said salt is NaCl.

107. The high ionic strength gel loading solution of claim 105, wherein said salt is a tetramethylalkyl salt.

108. A high ionic strength gel loading solution according to any one of claims 105 to 107, for use in admixing with a sample suspected of containing cleaved RNA.

109. A high ionic strength gel loading solution according to any one of claims 105 to 108, for use in an assay to detect base pair mismatches in double stranded nucleic acids.

110. A high ionic strength gel loading solution according to any one of claims 105 to 109, for use in an assay to detect base pair mismatches in double stranded nucleic acids in which nucleic acid cleavage products are separated by gel electrophoresis under conditions that allow the cleavage products to remain double-stranded.

111. A method of screening for an RNase mismatch cleavage activity enhancing agent, comprising the steps of:

(a) obtaining a digested protein mixture;

(b) fractionating said digested protein mixture into two or more fractions; and

(c) testing a fraction of the digested protein mixture for the ability to enhance RNase mismatch cleavage activity of an RNase cleavage reaction mixture.

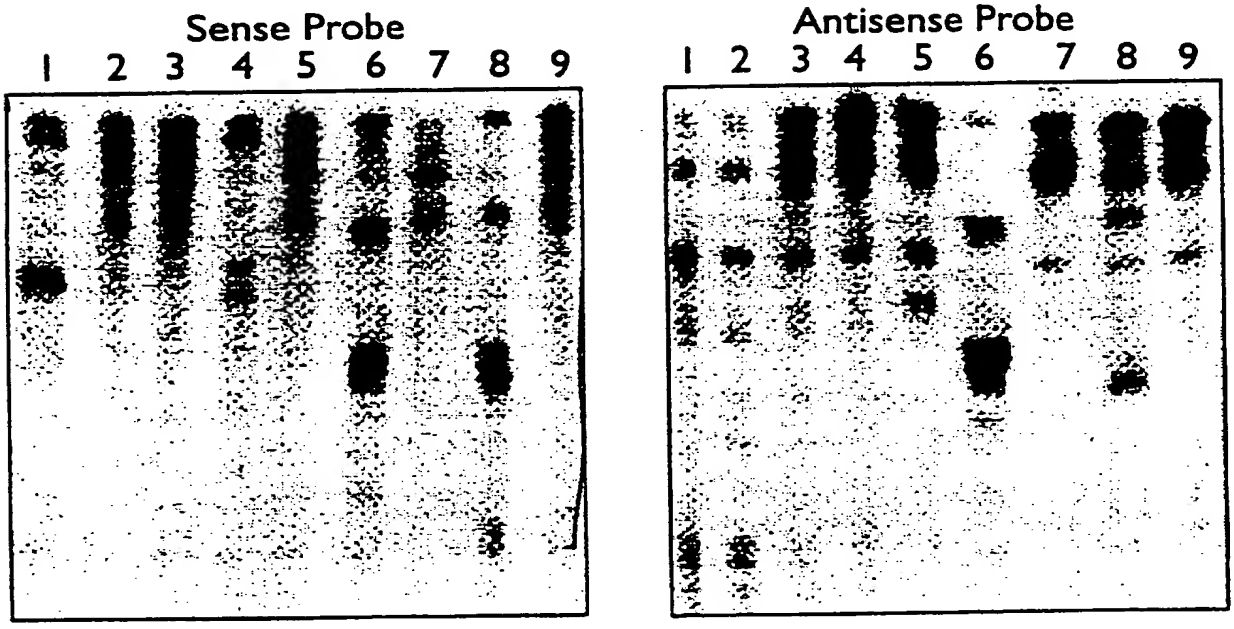
112. The method of claim 111, wherein step (c) comprises the steps of:

(a) preparing a test RNA digestion buffer comprising the fraction and an RNase enzyme;

(b) preparing a control RNA digestion buffer substantially identical to the test RNA digestion buffer but lacking said fraction; and

(c) performing parallel tests with both said test RNA digestion buffer and said control RNA digestion buffer to determine the relative activities of the RNase enzyme in said test and control buffers.

FIG. 1



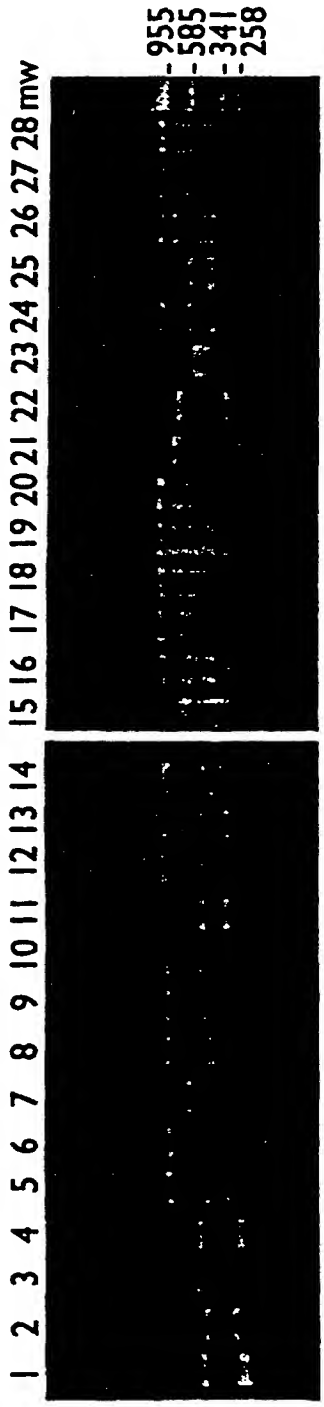

955
585
341
258
mw 28 27 26 25 24 23 22 21 20 19 18 17 16 15
14 13 12 11 10 9 8 7 6 5 4 3 2 1

FIG. 2

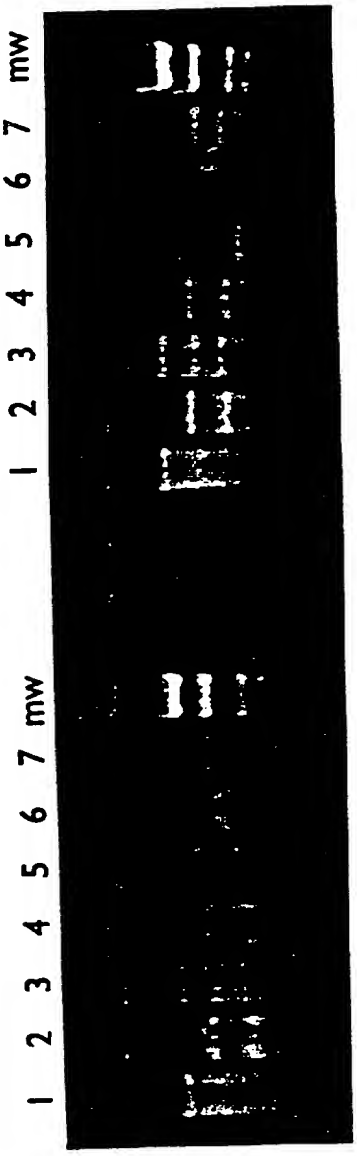
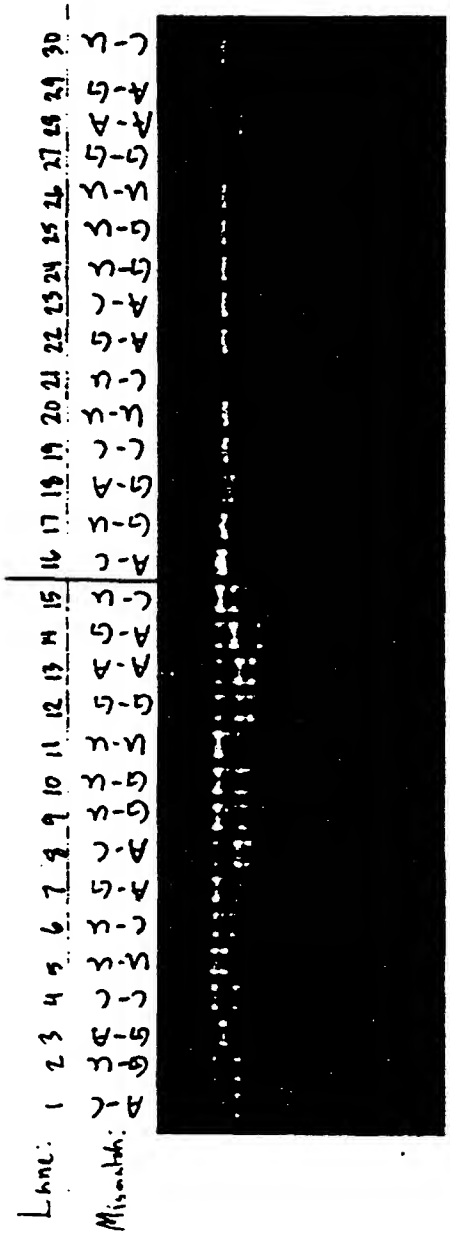


FIG. 3

FIG. 4


Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Mixture:

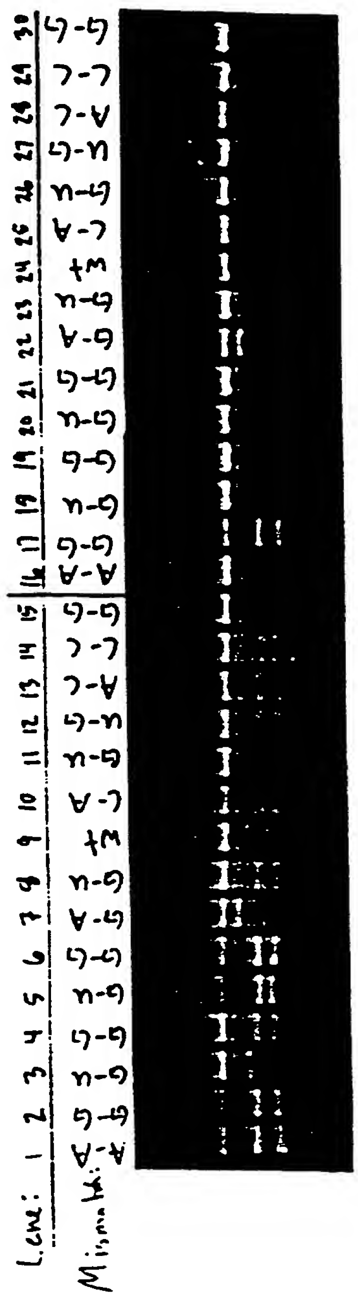


FIG. 5

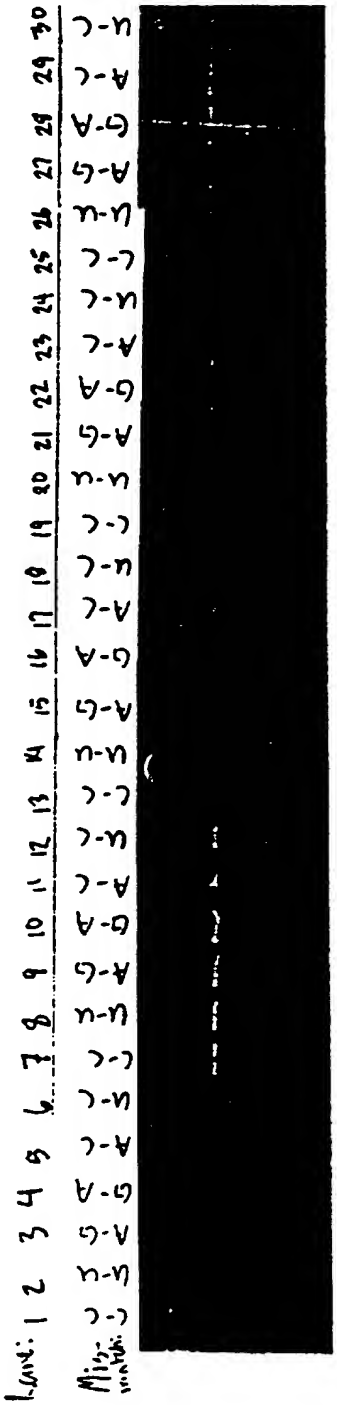

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Mis-match

FIG. 6

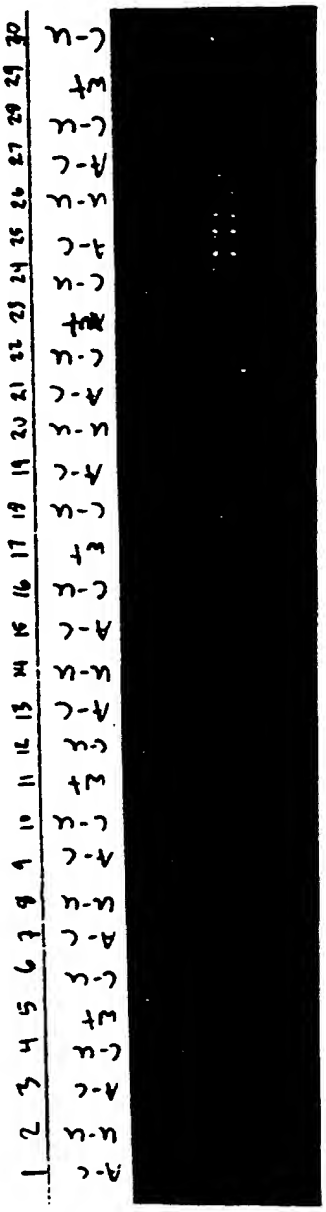


FIG. 7

FIG. 8A

C-C

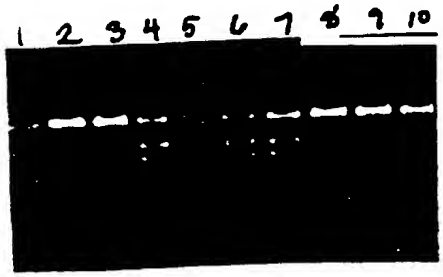


FIG. 8B

U-C

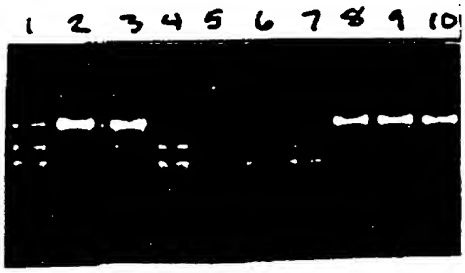


FIG. 8C

G-A

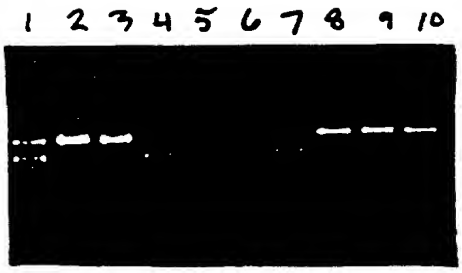


FIG. 8D

No Mismatch

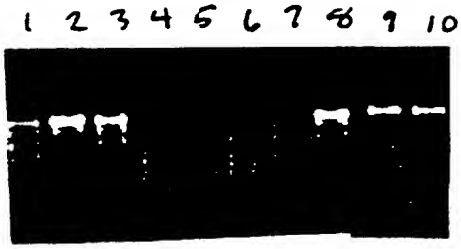


FIG. 8E

C-A

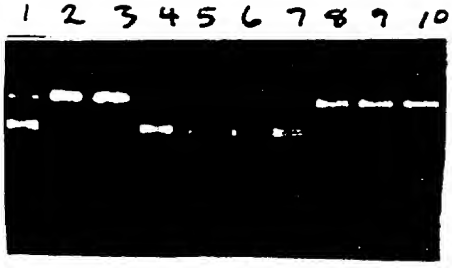


FIG. 9A

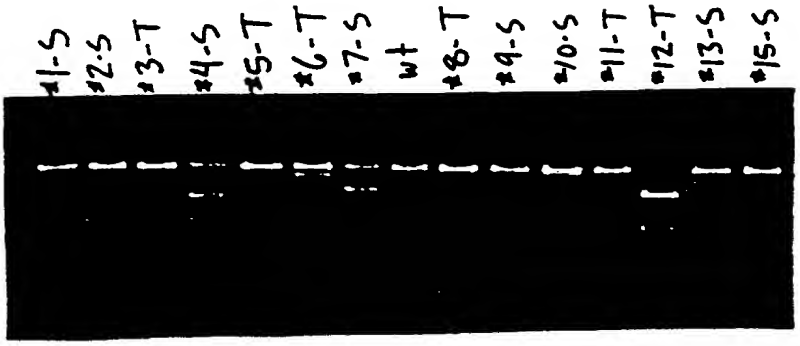


FIG. 9B

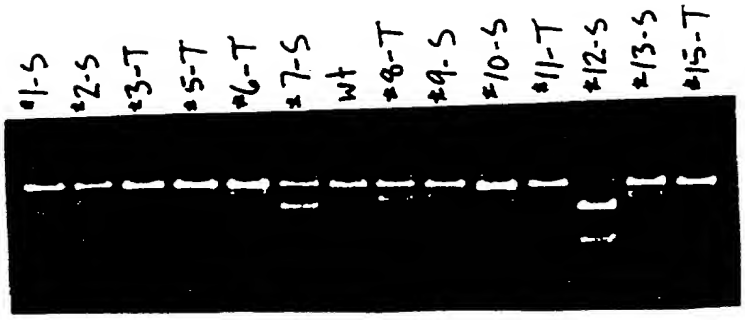


FIG. 9C

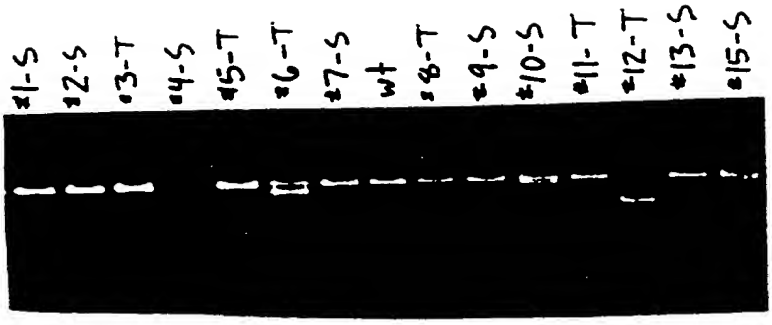
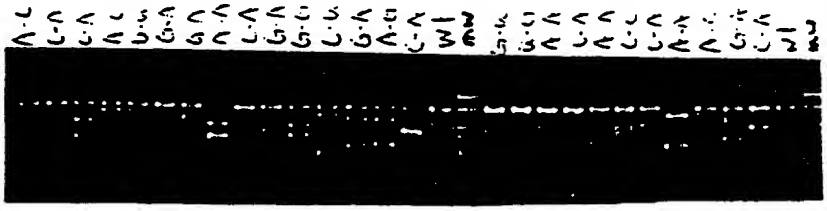
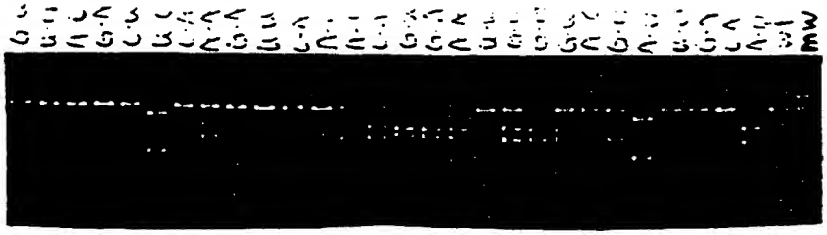


FIG. 10A



RNase A RNase 1

FIG. 10B



RNase T1

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 96/00073

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12N9/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,4 946 773 (MANIATIS T. ET AL) 7 August 1990 cited in the application see the whole document	1-6, 14-16, 18, 25-56,58
A	WO,A,91 15600 (CITY OF HOPE) 17 October 1991 see the whole document	1-6, 14-16, 18, 25-56,58
A	WO,A,93 20233 (UNIVERSITY OF MARYLAND AT BALTIMORE) 14 October 1993 see the whole document	1-6, 14-16, 18, 25-56,58

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 June 1996	27.06.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Osborne, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern· ·al Application No
PCT/US 96/00073

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 329 311 (MEDICAL RESEARCH COUNCIL) 23 August 1989 see page 1, line 10 - line 31	1
A	GB,A,2 179 735 (LIFECODES CORP.) 11 March 1987 see the whole document	1,22

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir. ational application No.
PCT/US96/00073

Box I Observations where certain claims were found unsearchable (Continuation f item 1 of first sheet)

This internati nal search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

CLAIMS 81-90 CONCERN A NUCLEIC ACID DIGESTION BUFFER PER SE. THE SEARCH DIV ISION CONSIDERS THAT A COMPLETE SEARCH FOR SUCH A BUFFER IS NOT ECONOMICALL Y FEASIBLE AND THEREFORE IT HAS BEEN RESTRICTED TO THOSE CLASSES COVERING T HE USE OF THE BUFFER OF CLAIMS 81-90 IN THE METHOD CLAIMS WHICH RELATE ./.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

TO THE CLEAVAGE OF A DOUBLE -STRANDED NUCLEIC ACID MOLECULE THAT CONTAINS A BASE PAIR MISMATCH.

INTERNATIONAL SEARCH REPORT

...rmation on patent family members

Intern ...al Application No
PCT/US 96/00073

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4946773	07-08-90	NONE	
WO-A-9115600	17-10-91	AU-B- 7762091	30-10-91
WO-A-9320233	14-10-93	AU-B- 3919293	08-11-93
EP-A-329311	23-08-89	JP-A- 2104300	17-04-90
		US-A- 5217863	08-06-93
GB-A-2179735	11-03-87	US-A- 4794075	27-12-88
		BE-A- 905348	02-03-87
		CA-A- 1286618	23-07-91
		DE-A- 3629190	05-03-87
		FR-A,B 2586705	06-03-87
		JP-A- 62158499	14-07-87